PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, 15/54, 9/10, 5/10, A01H 5/00 | A1

(11) International Publication Number: WO 00/04173

(43) International Publication Date: 27 January 2000 (27.01.00)

(21) International Application Number: PCT/EP99/04940

(22) International Filing Date: 12 July 1999 (12.07.99)

(30) Priority Data:
09/118,276 17 July 1998 (17.07.98) US

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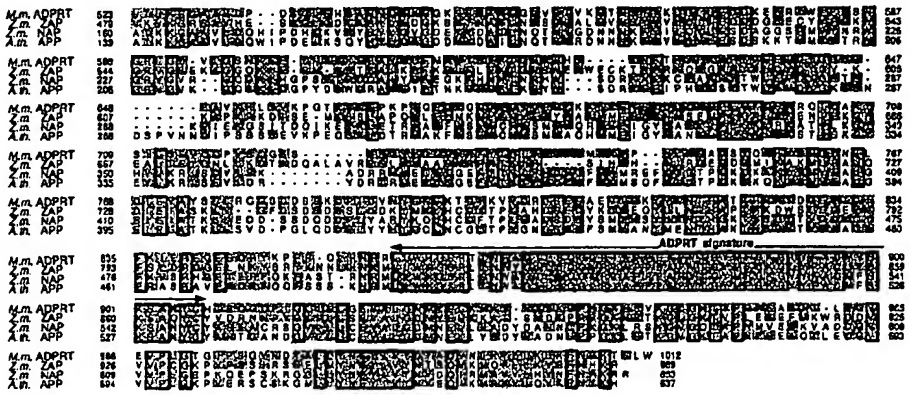
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS AND MEANS TO MODULATE PROGRAMMED CELL DEATH IN EUKARYOTIC CELLS



(57) Abstract

Means and methods are provided to modulate programmed cell death (PCD) in eukaryotic cells and organisms, particularly plant cells and plants, by introducing of "PCD modulating chimeric genes" influencing the expression and/or apparent activity of endogenous poly-ADP-ribose polymerase (PARP) genes. Programmed cell death may be inhibited or provoked. The invention particularly relates to the use of nuleotide sequences encoding proteins with PARP activity for modulating PCD, for enhancing growth rate of for producing stress tolerant cells and organisms.

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Methods And Means To Modulate Programmed Cell Death In Eukaryotic Cells.

Field of the invention

The invention relates to the use of poly (ADP-ribose) polymerase (PARP) proteins, particularly mutant PARP proteins or parts thereof, and genes encoding the same, to produce eukaryotic cells and organisms, particularly plant cells and plants, with modified programmed cell death. Eukaryotic cells and organisms, particularly plant cells and plants, are provided wherein either in at least part of the cells, preferably selected cells, the programmed cell death (PCD) is provoked, or wherein, on the contrary, PCD of the cells or of at least part of the cells in an organism is inhibited, by modulation of the level or activity of PARP proteins in those cells. The invention also relates to eukaryotic cells and organisms, particularly plant cells and plants, expressing such genes.

Description of related art

Programmed cell death (PCD) is a physiological cell death process involved in the elimination of selected cells both in animals and in plants during developmental processes or in response to environmental cues (for a review see Ellis *et al.* 1991; Pennell and Lamb, 1997). The disassembly of cells undergoing PCD is morphologically accompanied by condensation, shrinkage and fragmentation of the cytoplasm and nucleus, often into small sealed packets (Cohen 1993, Wang *et al.* 1996). Biochemically, PCD is characterized by fragmentation of the nuclear DNA into generally about 50 kb fragments representing oligonucleosomes, as well as the induction of cysteine proteinases and endonucleases. The fragmentation of the DNA can be detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of DNA 3'-OH groups in sections of cells. (Gavrieli *et al.* 1992). Cell death by PCD is clearly distinct from cell death by necrosis, the latter involving cell swelling, lysis and leakage of the cell contents.

In animals, PCD is involved in the elimination or death of unwanted cells such as tadpole tail cells at metamorphosis, cells between developing digits in vertebrates,

overproduced vertebrate neurons, cells during cell specialization such as keratocytes etc. Damaged cells, which are no longer able to function properly, can also be eliminated by PCD, preventing them from multiplying and/or spreading. PCD, or the lack thereof, has also been involved in a number of pathological conditions in humans (AIDS, Alzheimer's disease, Huntington's disease, Lou Gehring's disease, cancers).

In plants, PCD has been demonstrated or is believed to be involved in a number of developmental processes such as e.g., removal of the suspensor cells during the development of an embryo, the elimination of aleurone cells after germination of monocotyledonous seeds; the elimination of the root cap cells after seed germination and seedling growth; cell death during cell specialization as seen in development of xylem tracheary element or trichomes, or floral organ aborting in unisexual flowers. Also the formation of aerochyma in roots under hypoxic conditions and the formation of leaf lobes or perforations in some plants seem to involve PCD. Large scale cell death in plants occurs during upon senescence of leaves or other organs. The hypersensitive response in plants, in other words the rapid cell death occurring at the site of entry of an avirulent pathogen leading to a restricted lesion, is an another example of PCD in response to an environmental cue.

Animal or plant cells dying in suspension cultures, particularly in low-density cell suspension cultures, also demonstrate the characteristics of PCD.

An enzyme which has been implied to be involved in PCD or apoptosis is poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase (PARP), also known as poly(ADP-ribose) transferase (ADPRT) (EC 2.4.2.30), is a nuclear enzyme found in most eukaryotes, including vertebrates, arthropods, molluscs, slime moulds, dinoflagellates, fungi and other low eukaryotes with the exception of yeast. The enzymatic activity has also been demonstrated in a number of plants (Payne *et al.*, 1976; Willmitzer and Wagner, 1982; Chen *et al.*, 1994; O'Farrell, 1995).

PARP catalyzes the transfer of an ADP-ribose moiety derived from NAD^+, mainly to the carboxyl group of a glutamic acid residue in the target protein, and subsequent ADP-ribose polymerization. The major target protein is PARP itself, but also histones,

high mobility group chromosomal proteins, a topoisomerase, endonucleases and DNA polymerases have been shown to be subject to this modification.

The PARP protein from animals is a nuclear protein of 113-120 kDa, abundant in most cell types, that consist of three major functional domains: an amino-terminal DNA-binding domain containing two Zn-finger domains, a carboxy-terminal catalytic domain, and an internal domain which is automodified (de Murcia and Ménissier de Murcia, 1994; Kameshita *et al.*, 1984; Lindahl *et al.*, 1995). The enzymatic activity *in vitro* is greatly increased upon binding to single-strand breaks in DNA. The *in vivo* activity is induced by conditions that eventually result in DNA breaks (Alvarez-Gonzalez and Althaus, 1989; Ikejima *et al.*, 1990). Automodification of the central domain apparently serves as a negative feedback regulation of PARP.

PARP activity in plant cells was first demonstrated by examining the incorporation of ^{3}H from labelled NAD^{+} into the nuclei of root tip cells (Payne *et al.*, 1976; Willmitzer and Wagner, 1982). The enzymatic activity was also partially purified from maize seedlings and found to be associated with a protein of an apparent molecular mass of 113 kDa, suggesting that the plant PARP might be similar to the enzyme from animals (Chen *et al.*, 1994; O'Farrell, 1995).

cDNAs corresponding to PARP proteins have isolated from several species including mammals, chicken, *Xenopus*, insects and *Caenorhabditis elegans*.
Chen *et al.* (1994) have reported PARP activity in maize nuclei and associated this enzymatic activity with the presence of an approximately 114 kDa protein present in an extract of maize nuclei. O' Farrel (1995) reported that RT-PCR-amplification on RNA isolated from maize (using degenerate primers based on the most highly conserved sequences) resulted in a 300 bp fragment, showing 60% identity at the amino acid level with the human PARP protein. Lepiniec *et al.* (1995) have isolated and cloned a full length cDNA from *Arabidopsis thaliana* encoding a 72 kDa protein with high similarity to the catalytic domain of vertebrate PARP. The N-terminal domain of the protein does not reveal any sequence similarity with the corresponding domain of PARP from vertebrates but is composed of four stretches of amino acids (named A1, A2, B and C) showing similarity to the N-terminus of a number of nuclear and

DNA binding proteins. The predicted secondary structure of A1 and A2 was a helix-loop-helix structure.

The Genbank database contains the sequences of two cDNAS from *Zea mays* for which the amino acid sequence of the translation products has either homology to the conventional PARP proteins (AJ222589) or to the non-conventional PARP proteins, as identified in *Arabidopsis* (AJ222588)

The function(s) of PARP and poly-ADP ribosylation in eukaryotic cells is (are) not completely clear. PARP is involved or believed to be involved either directly or indirectly in a number of cellular processes such as DNA repair, replication and recombination, in cell division and cell differentiation or in the signalling pathways that sense alterations in the integrity of the genome. As PARP activity may significantly reduce the cellular NAD^+ pool, it has also been suggested that the enzyme may play a critical role in programmed cell death (Heller *et al.*, 1995; Zhang *et al.*, 1994). Further, it has been suggested that nicotinamide resulting from NAD+ hydrolysis or the products of the turn-over of poly-ADP-ribose by poly-ADP-ribose glycohydrolase may be stress response signals in eukaryotes.

The information currently available on the biological function of plant PARP has come from experiments involving PARP inhibitors suggesting an *in vivo* role in the prevention of homologous recombination at sites of DNA damage as rates of homologous intrachromosomal recombination in tobacco are increased after application of 3-aminobenzamide (3ABA) (Puchta *et al.*, 1995). Furthermore, application of PARP inhibitors, such as 3ABA, nicotinamide, and 6(5H)-phenasthridinone, to differentiating cells of *Zinnia* or of *Helianthus tuberosum* has been shown to prevent development of tracheary elements (Hawkins and Phillips, 1983; Phillips and Hawkins, 1985; Shoji *et al.*, 1997; Sugiyama *et al.*, 1995), which is considered to be an example of programmed cell death in plants.

PCT application WO97/06267 describes the use of PARP inhibitors to improve the transformation (qualitatively or quantitatively) of eukaryotic cells, particularly plant cells.

Lazebnik *et al.* (1994) identified a protease with properties similar to the interleukin 1-β-converting enzyme capable of cleaving PARP, which is an early event in apoptosis of animal cells.

Kuepper *et al.* (1990) and Molinette *et al.* (1993) have described the overproduction of the 46 kDa human PARP DNA-binding domain and various mutant forms thereof, in transfected CV-1 monkey cells or human fibroblasts and have demonstrated the trans-dominant inhibition of resident PARP activity and the consequent block of base excision DNA repair in these cells.

Ding *et al.* (1992), and Smulson *et al.* (1995) have described depletion of PARP by antisense RNA expression in mammalian cells and observed a delay in DNA strand break joining, and inhibition of differentiation of 3T3-L1 preadipocytes.

Ménissier de Murcia *et al.*, (1997) and Wang *et al.* (1995, 1997) have generated transgenic "knock-out" mice mutated in the PARP gene, indicating that PARP is not an essential protein. Cells of PARP-deficient mice are, however, more sensitive to DNA damage and differ from normal cells of animals in some aspects of induced cell death (Heller *et al.*, 1995).

Summary and objects of the invention.

The invention provides a method for modulating programmed cell death in a eukaryotic cell, comprising reducing the functional level of the total PARP activity in a eukaryotic cell using the nucleotide sequence of a PARP gene of the ZAP class, and the nucleotide sequence of a PARP gene of the NAP class, preferably to reduce expression of the endogeneous PARP genes, to reduce the apparent activity of the proteins encoded by the endogenous PARP genes or to alter the nucleotide sequence of the endogenous PARP genes.

The invention also provides a method for modulating programmed cell death in a eukaryotic cell, comprising introducing a first and a second PCD modulating chimeric gene in a eukaryotic cell, preferably a plant cell, wherein the first PCD modulating

chimeric gene comprises the following operably linked DNA regions: a promoter, operative in a eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a Zn-finger containing PARP protein of the ZAP class; or is capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of ZAP class and a DNA region involved in transcription termination and polyadenylation

and wherein the second PCD modulating chimeric gene comprises the following operably linked DNA regions :a promoter, operative in the eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a PARP protein of the NAP class; or capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of the NAP class, and a DNA region involved in transcription termination and polyadenylation; and wherein the total apparent PARP activity in the eukaryotic cell is reduced significantly, (preferably the total apparent PARP activity is reduced from about 75% to about 90% of the normal apparent PARP activity in the eukaryotic cell, and the eukaryotic cell is protected against programmed cell death) or almost completely (preferably the total apparent PARP activity is reduced from about 90% to about 100% of the normal apparent PARP activity in the eukaryotic cell, and the cell is killed by programmed cell death).

Preferably the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode a sense RNA molecule which is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In an alternative method for modulating programmed cell death, provided by the invention, the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode an RNA molecule which is capable of reducing the expression of said endogenous PARP gene of the ZAP or the NAP class.

In yet an alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP or the NAP class and the RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the mRNA resulting from transcription of the endogenous PARP gene of the ZAP or the NAP class, wherein the sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein that RNA molecule is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In a further alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP or the NAP class, preferably comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138 or comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370, the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11.

The promoter of the first and second chimeric PCD modulating genes, or both, may be a tissue specific or inducible promoter such as a promoter is selected from a fungus-responsive promoter, a nematode-responsive promoter, an anther-selective promoter, a stigma-selective promoter, a dehiscence-zone selective promoter.

The invention also provides a method for modulating programmed cell death in a plant cell, comprising introduction of a PCD modulating chimeric gene in said plant cell, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter, a DNA region, which when

transcribed yields a RNA molecule, which is either capable of reducing the expression of endogenous PARP genes; or is capable of being translated into a peptide or protein which when expressed reduces the apparent PARP activity in the plant cell, and a DNA region involved in transcription termination and polyadenylation, wherein the total apparent PARP activity in the plant cell is reduced from about 75% to about 100% of the normal apparent PARP activity in the plant cell.

It is another objective of the invention to provide the first and second chimeric PCD modulating gene as well as a eucaryotic cell, particularly a plant cell comprising the first and second chimeric PCD modulating gene and non-human eukaryotic organisms, particularly plants comprising such cells.

It is yet another objective of the invention to provide a method for modulating programmed cell death in cells of a plant, comprising introducing a PCD modulating chimeric gene in the cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and a DNA region involved in transcription termination and polyadenylation.

The invention also relates to a method for increasing the growth rate of a plant, comprising introducing a PCD modulating chimeric gene in cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule, being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and a DNA region involved in transcription termination and polyadenylation.

It is another objective of the invention to provide a method for producing stress tolerant cells of a plant comprising introducing a PCD modulating chimeric gene into cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule, RNA molecule being capable of reducing

the expression of an endogenous PARP gene of the ZAP class: and a DNA region involved in transcription termination and polyadenylation.

The invention also relates to the use of a nucleotide sequence encoding a protein with PARP activity, preferably a PARP protein of the ZAP class, to modulate programmed cell death in a plant cell or plant or to produce a stress tolerant plant cell or plant or to increase the growth rate of a plant cell or plant.

Brief description of the drawings

Figure 1. The deduced N-terminal amino acid sequences of plant poly(ADP-ribose) polymerases.

(A) Alignment of the sequences upstream of the NAD^+-binding domain found in *Arabidopsis thaliana* APP (*A.th.* APP; EMBL accession number Z48243; SEQ ID No 6) and the maize homolog NAP (*Z.m.* NAP; EMBL accession number AJ222588; SEQ ID No 4). The domain division shown is as previously proposed (Lepiniec *et al.*, 1995). The nuclear localization signal (NLS) located in the B domain is indicated by the bracket. The sequence of the B domain is not very well conserved between dicotyledonous and monocotyledonous plants. The C domain is probably comparable in function to the automodification domain of PARP from animals. The imperfect repeats, A1 and A2, are also present in maize NAP. To illustrate the internally imperfect two-fold symmetry within the repeat sequence, the properties of amino acid residues are highlighted below the sequences as follows: filled-in circles, hydrophobic residue; open circle, glycine; (+), positively charged residue; (-), negatively charged residue; wavy line, any residue. The axis of symmetry is indicated by the vertical arrowhead and arrowhead lines mark the regions with the inverted repetition of amino acid side chain properties.

(B) Alignment of the DNA-binding and auto-catalytic domains of mouse PARP and maize ZAP. Zn-finger-containing maize ZAP1 and ZAP2 (partial cDNA found by the 5'RACE PCR analysis) are indicated as *Z.m.* ZAP (EMBL accession number AJ222589; SEQ ID No 2) and *Z.m.* ZAP(race) (SEQ ID No 11 from amino acid at position 1 to amino acid at position 98), respectively, and the mouse PARP, *M.m.* ADPRT (Swissprot accession number P11103). The Zn-fingers and bipartite NLS of the mouse enzyme are indicated by brackets, the Caspase 3 cleavage site by

the asterisk, and the putative NLS in the ZAP protein by the bracket in bold below the maize sequence. The amino acid residues that are conserved in all sequences are boxed; amino acid residues with similar physico-chemical properties are shaded with the uppermost sequence as a reference.

Figure 2. Comparison of the NAD+-binding domain of mouse PARP and plant PARP proteins. The range of the "PARP signature" is indicated above the sequences. Names and sequence alignment are as in Figure 1.

Figure 3. Estimation of the gene copy number and transcript size for the *nap* and *zap* genes.

(A) and (B) Maize genomic DNA of variety LG2080 digested with the indicated restriction endonucleases, resolved by agarose gel electrophoresis, blotted, and hybridized with radioactively labelled DNA probes prepared from the 5' domains of the *nap* and *zap* cDNA, which do not encode the NAD^+-binding domain. The hybridization pattern obtained with the *nap* probe (A) is simple and indicates a single *nap* gene in the maize genome. As can be seen from the hybridization pattern (B), there might be at least two *zap* genes. To determine the size of the transcripts encoded by the *zap* and *nap* genes, approximately 1 µg of poly(A)$^+$ RNA extracted from roots (lane 1) and shoots (lane 2) of 6-day-old seedlings were resolved on an agarose gel after denaturation with glyoxal, blotted, and hybridized with *nap* (C) and *zap* (D) ^{32}P-labelled cDNA. ^{33}P 5' end-labelled *Bsf*EII fragments of λDNA were used as a molecular weight markers in both DNA and RNA gel blot experiments; their positions are indicated in kb to the left of each panel.

Figure 4. Analysis of APP expression in yeast.

(A) Schematic drawing of the expression cassette in pV8SPA. The expression of the app cDNA is driven by a chimeric yeast promoter, which consists of the minimal TATA box-containing promoter region of the cyc1 gene (CYC1) and an upstream activating promoter region of the ga110 gene (GAL10), the latter providing promoter activation by galactose. Downstream regulatory sequences are derived from the gene encoding phosphoglycerol kinase (3PGK) (Kuge and Jones, 1994). The app-coding region is drawn with a division in putative domains as proposed earlier (Lepiniec *et al.*, 1995): A1 and A2 correspond to imperfect 27-

amino acid repeats, in between which there is a sequence (B domain), rich in positively charged amino acids and resembling the DNA-binding domains of a number of DNA-binding proteins. The amino acid sequence of the B domain is shown below the map and the stretch of arginine and lysine residues, which may function as an NLS is drawn in bold. Methionine residues (M^1, M^{72}), which may function as translation initiation codons, are indicated above the map. The C domain is rich in glutamic acid residues, resembling in its composition, but not in its sequence, the auto-modification domain of PARP from animals.

(B) Immunoblot (Western blot) and Northern blot analyses of the DY (pYeDP1/8-2) and DY(pV8SPA) strains, indicated as (vector) and (app), respectively. Strains were grown in SDC medium supplemented with glucose (GLU), galactose (GAL), galactose and 3mM of 3ABA (GAL+3ABA), or galactose and 5 mM nicotinamide (GAL+NIC). Total RNA or total protein were extracted from the same cultures. Ten micrograms of total protein were fractionated by electrophoresis on 10% SDS-PAGE, electroblotted, and probed with anti-APP antisera. Five micrograms of total RNA were resolved by electrophoresis on an 1.5% agarose gel, blotted onto nylon membranes, and hybridized with ^{32}P-labeled DNA fragments derived from the app cDNA. Positions of the molecular weight marker bands are indicated to the left in kilobases (kb) and kilodalton (kDa).

Figure 5. Poly(ADP-ribose) polymerase activity of the APP protein.

(A) The total protein extracts were prepared from DY(pYeDP1/8-2) grown on SDC with 2% galactose (vector GAL) and DY(pV8SPA) grown either on SDC with 2% glucose (app GLU), on SDC with 2% galactose (app GAL), or on SDC with 2% galactose and 3 mM 3ABA (app GAL+3ABA). To detect the synthesis of the poly(ADP-ribose) in these extracts, samples were incubated with ^{32}P-NAD^+ for 40 min at room temperature. Two control reactions were performed: 100 ng of the purified human PARP were incubated either in a reaction buffer alone (PARP) (lane 5), or with protein extract made from DY(pYeDP1/8-2) culture grown on glucose (vector GLU+PARP) (lane 6). The autoradiograph obtained after exposure of the dried gel to X-Omat Kodak film is shown. ORi corresponds to the beginning of the sequencing gel.

(B) Stimulation of poly(ADP-ribose) synthesis by DNA in protein extracts from DY(pV8SPA). Amounts of sonicated salmon sperm DNA added to the nucleic acid

depleted yeast extracts are indicated in $\mu g\ ml^{-1}$. The synthesis of the poly(ADP-ribose) is blocked by 3ABA, which was added in one of the reactions at a concentration of 3 mM (lane 5). To ensure the maximal recovery of the poly(ADP-ribose), 20 µg of glycogen were included as a carrier during precipitation steps; this, as can be seen, however resulted in high carry-over of the unincorporated label.

Figure 6. Schematic representation of the T-DNA vectors comprising the PCD modulating chimeric genes of the invention. P35S: CaMV35S promoter; L: cab22 leader; ZAP; coding region of a PARP gene of the ZAP class; 5'ZAP: N-terminal part of the coding regon of a PARP gene of the ZAP class in inverted orientation; 3' 35S: CaMV35S 3' end transcription termination signal and polyadenylation signal; pACT2: promoter region of the actin gene; pNOS; nopaline synthase gene promoter; gat: gentamycin acetyl transferase; bar: phosphinotricin acetyl transferase; 3'NOS: 3' end transcription termination signal and polyadenylation signal of nopaline synthase gene; APP: coding region of a PARP gene of the NAP class; 5'APP: N-terminal part of the coding regon of a PARP gene of the NAP class in inverted orientation ; LB: left T-DNA border; RB: right T-DNA border; pTA29: tapetum specific promoter, pNTP303: pollen specific promoter.

Detailed description of preferred embodiments

For the purpose of the invention, the term "plant-expressible promoter" means a promoter which is capable of driving transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, e.g., certain promoters of viral or bacterial origin such as the CaMV35S or the T-DNA gene promoters.

The term "expression of a gene" refers to the process wherein a DNA region under control of regulatory regions, particularly the promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another

nucleic acid or protein or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA or a ribozyme. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide.

The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell under control of suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. An endogenous plant gene is a gene which is naturally found in a plant species. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory regions of the gene.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

The invention is based on the one hand on the finding that eukaryotic cells, particularly plant cells, quite particularly *Zea mays* cells contain simultaneously at least two functional major PARP protein isoforms(classes) which differ in size and amino-acid sequence, yet are both capable of binding DNA, particularly DNA with single stranded breaks, and both have poly-ADP ribosylation activity. On the other hand, the inventors have realized that programmed cell death in eukaryotes, particularly in plants, can be modulated by altering the expression level of the PARP genes or by altering the activity of the encoded proteins genetically, and that in order

to achieve this goal, the expression of both genes needs to be altered or in the alternative both classes of proteins need to be altered in their activity.

It is clear that the failure of the art to show that eukaryotic cells, particularly plant cells, comprise two functional isoforms of PARP proteins, encoded by different classes of genes, has hampered efficient modulation of PARP activity in those cells by recombinant DNA methods. Various embodiments of the methods and means are represented by the description, the Examples and the claims.

Thus, the invention relates to modulation -i.e. the enhancement or the inhibition- of programmed cell death or apoptosis in eukaryotic cells, preferably plant cells, by altering the level of expression of PARP genes, or by altering the activity or apparent activity of PARP proteins in that eukaryotic cell. Conveniently, the level of expression of PARP genes or the activity of PARP proteins is controlled genetically by introduction of PCD modulating chimeric genes altering the expression of PARP genes and/or by introduction of PCD modulating chimeric genes altering the apparent activity of the PARP proteins and/or by alteration of the endogenous PARP encoding genes.

As used herein, "enhanced PCD" with regard to specified cells, refers to the death of those cells, provoked by the methods of the invention, whereby the killed cells were not destined to undergo PCD when compared to similar cells of a normal plant not modified by the methods of the invention, under similar conditions.

"Inhibited PCD" with regard to specified cells is to be understood as the process whereby a larger fraction of those cells or groups of cells, which would normally (without the intervention by the methods of this invention) undergo programmed cell death under particular conditions, remain alive under those conditions.

The expression of the introduced PCD modulating chimeric genes or of the modified endogenous genes will thus influence the functional level of PARP protein, and indirectly interfere with programmed cell death. A moderate decrease in the functional level of PARP proteins leads to an inhibition of programmed cell death,

particularly to prevention of programmed cell death, while a severe decrease in the functional level of the PARP proteins leads to induction of programmed cell death.

In accordance with the invention, it is preferred that in order to inhibit or prevent programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased significantly, however avoiding that DNA repair (governed directly or indirectly by PARP) is inhibited in such a way that the cells wherein the function of the PARP proteins is inhibited cannot recover from DNA damage or cannot maintain their genome integrity. Preferably, the level and/or activity of the PARP proteins in the target cells, should be decreased about 75 %, preferably about 80%, particularly about 90% of the normal level and/or activity in the target cells so that about 25%, preferably about 20%, particularly about 10% of the normal level and/or acttivity of PARP is retained in the target cells . It is further thought that the decrease in level and/or activity of the PARP proteins should not exceed 95%, preferably not exceed 90% of the normal activity and/or level in the target cells. Methods to determine the content of a specific protein such as the PARP proteins are well known to the person skilled in the art and include, but are not limited to (histochemical) quantification of such proteins using specific antibodies. Methods to quantify PARP activity are also available in the art and include the above-mentioned TUNEL assay (*in vivo*) or the *in vitro* assay described Collinge and Althaus (1994) for synthesis of poly (ADP-ribose) (see Examples).

Also in accordance with the invention, it is preferred that in order to trigger programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased substantially, preferably reduced almost completely such that the DNA repair and maintenance of the genome integrity are no longer possible. Preferably, the combined level and/or activity of the PARP proteins in the target cells, should be decreased at least about 90%, preferably about 95%, more preferably about 99%, of the normal level and/or activity in the target cells, particularly the PARP activity should be inhibited completely. It is particularly preferred that the functional levels of both classes of PARP proteins seperately are reduced to the mentioned levels.

For the purpose of the invention, PARP proteins are defined as proteins having poly (ADP-ribose) polymerase activity, preferably comprising the so-called "PARP signature". The PARP signature is an amino acid sequence which is highly conserved between PARP proteins, defined by de Murcia and Menussier de Murcia (1994) as extending from amino acid at position 858 to the amino acid at position 906 from the *Mus musculus* PARP protein. This domain corresponds to the amino acid sequence from position 817 to 865 of the conventional PARP protein of *Zea mays* (ZAP1; SEQ ID No 2) or to the amino acid sequence from position 827 to 875 of the conventional PARP protein of *Zea mays* (ZAP2; SEQ ID No 11) or to the amino acid sequence from position 500 to 547 of the non-conventional PARP protein of *Zea mays* (SEQ ID No 4) or to the amino acid sequence from position 485 to 532 of the non-conventional PARP protein of Arabidopsis thaliana (SEQ ID No 6). This amino sequence is highly conserved between the different PARP proteins (having about 90% to 100% sequence identity). Particularly conserved is the lysine at position 891 (corresponding to position 850 of SEQ ID No 2, position 861 of SEQ ID No 11, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6) of the PARP protein from *Mus musculus*, which is considered to be involved in the catalytic activity of PARP proteins. Particularly the amino acids at position 865, 866, 893, 898 and 899 of the PARP protein of *Mus musculus* or the corresponding positions for the other sequences are variable. PARP proteins may further comprise an N-terminal DNA binding domain and/or a nuclear localization signal (NLS).

Currently, two classes of PARP proteins have been described. The first class, as defined herein, comprises the so-called classical Zn-finger containing PARP proteins (ZAP). These proteins range in size from 113-120 kDA and are further characterized by the presence of at least one, preferably two Zn-finger domains located in the N-terminal domain of the protein, particularly located within the about 355 to about 375 first amino acids of the protein. The Zn-fingers are defined as peptide sequences having the sequence $CxxCx_nHxxC$ (whereby n may vary from 26 to 30) capable of complexing a Zn atom. Examples of amino acid sequences for PARP proteins from the ZAP class include the sequences which can be found in the PIR protein database with accession number P18493 (*Bos taurus*), P26466 (*Gallus gallus*), P35875 (*Drosophila melanogaster*), P09874 (*Homo sapiens*), P11103 (*Mus musculus*), Q08824 (*Oncorynchus masou*), P27008 (*Rattus norvegicus*), Q11208

(*Sarcophaga peregrina*), P31669 (*Xenopus laevis*) and the currently identified sequences of the ZAP1 and ZAP2 protein from *Zea mays* (SEQ ID No 2 / SEQ ID No 11).

The nucleotide sequence of the corresponding cDNAs can be found in the EMBL database under accession numbers D90073 (*Bos taurus*), X52690 (*Gallus gallus*), D13806 (*Drosophila melanogaster*), M32721 (*Homo sapiens*), X14206 (*Mus musculus*), D13809 (*Oncorynchus masou*), X65496 (*Rattus norvegicus*), D16482 (*Sarcophaga peregrina*), D14667 (*Xenopus laevis*) and in SEQ ID No 1 and 10 (*Zea mays*).

The second class as defined herein, comprises the so-called non-classical PARP proteins (NAP). These proteins are smaller (72-73 kDa) and are further characterized by the absence of a Zn-finger domain at the N-terminus of the protein, and by the presence of an N-terminal domain comprising stretches of amino acids having similarity with DNA binding proteins. Preferably, PARP protein of these class comprise at least one amino acid sequence of about 30 to 32 amino acids which comprise the sequence R G x x x x G x K x x x x x R L (amino acids are represented in the standard one-letter code, whereby x stands for any amino acid; SEQ ID No 7). Even more preferably these PARP proteins comprise at least 1 amino acid sequence of about 32 amino acids having the sequence x L x V x x x R x x L x x R G L x x x G V K x x L V x R L x x A I (SEQ ID No 8) (the so-called A1 domain) or at least 1 amino acid sequence of about 32 amino acids having the sequence G M x x x E L x x x A x x R G x x x x G x K K D x x R L x x (SEQ ID No 9) (the so-called A2 domain) or both. Particularly, the A1 and A2 domain are capable of forming a helix-loop-helix structure. These PARP proteins may further comprise a basic "B" domain (K/R rich amino acid sequence of about 35 to about 56 amino acids, involved in targeting the protein to the nucleus) and/or a an acid "C" domain (D/E rich amino acid sequence of about 36 amino acids). Examples of protein sequences from the NAP class include the APP protein from *Arabidopsis thaliana* (accessible from PIR protein database under accession number Q11207; SEQ ID No 6) and the NAP protein from *Zea mays* (SEQ ID No 4). The sequence of the corresponding cDNAs can be found in the EMBL database under accession number Z48243 (SEQ ID No 5) and in SEQ ID No 3. That the second class of PARP proteins are indeed functional PARP proteins, i.e.

are capable of catalyzing DNA dependent poly(ADP-ribose) polymerization has been demonstrated by the inventors (see Example 2).

The inventors have further demonstrated that eukaryotic cells, particularly plant cells, express simultaneously genes encoding PARP proteins from both classes.

It is clear that for the purpose of the invention, other genes or cDNAs encoding PARP proteins from both classes as defined, or parts thereof, can be isolated from other eukaryotic species or varieties, particularly from other plant species or varieties. These PARP genes or cDNAs can be isolated e.g. by Southern hybridization (either low-stringency or high-stringency hybridization depending on the relation between the species from which one intends to isolate the PARP gene and the species from which the probe was ultimately derived) using as probes DNA fragments with the nucleotide sequence of the above mentioned PARP genes or cDNAs, or parts thereof, preferably parts which are conserved such as a gene fragment comprising the nucleotide sequence encoding the PARP signature mentioned supra. The nucleotide sequences corresponding to the PARP signature from the PARP proteins encoded by plant genes are the nucleotide sequence of SEQ ID No 1 from nucleotide 2558 to 2704 or the nucleotide sequence of SEQ ID No 3 from nucleotide 1595 to 1747 or the nucleotide sequence of SEQ ID No 5 from nucleotide 1575 to 1724. If a discrimination is to be made between the classes of PARP genes, parts of the PARP genes which are specific for the class, such as the N-terminal domains preceding the catalytic domain or parts thereof, should preferably be used.

Alternatively, the genes or cDNAs encoding PARP proteins or parts thereof, can also be isolated by PCR-amplification using appropriate primers such as the degenerated primers with the nucleotide sequence corresponding to the sequences indicated in SEQ ID No 13, SEQ ID No 14, or primers with the nucleotide sequence corresponding to the sequences indicated in SEQ ID No 15 to 20. However, it is clear that the person skilled in the art can design alternative oligonucleotides for use in PCR or can use oligonucleotides comprising a nucleotide sequence of at least 20, preferably at least about 30, particularly at least about 50, consecutive nucleotides of any of the PARP genes to isolate the genes or part thererof by PCR amplification.

It is clear that a combination of these techniques, or other techniques (including e.g. RACE-PCR), available to the skilled artisan to isolate genes or cDNAs on the basis of partial fragments and their nucleotide sequence, e.g. obtained by PCR amplification, can be used to isolate PARP genes, or parts thereof, suitable for use in the methods of the invention.

Moreover, PARP genes, encoding PARP proteins wherein some of the amino acids have been exchanged for other, chemically similar, amino acids (so-called conservative substitutions), or synthetic PARP genes (which encode similar proteins as natural PARP genes but with a different nucleotide sequence, based on the degeneracy of the genetic code) and parts thereof are also suited for the methods of the invention.

In one aspect of the invention, PCD in eukaryotic cells, particularly in plant cells, is inhibited by a moderate decrease in the functional level of PARP in those eukaryotic cells.

In one embodiment of this first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is moderately decreased.

In a particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of expression of the endogenous PARP genes. To this end, the transcribed DNA region encodes a biologically active RNA which decreases the mRNAs encoding NAP and ZAP class PARP proteins, that is available for translation. This can be achieved through techniques such as antisense RNA, co-suppression or ribozyme action.

As used herein, "co-suppression" refers to the process of transcriptional and/or post-transcriptional suppression of RNA accumulation in a sequence specific manner, resulting in the suppression of expression of homologous endogenous genes or transgenes.

Suppressing the expression of the endogenous PARP genes can thus be achieved by introduction of a transgene comprising a strong promoter operably linked to a DNA region whereby the resulting transcribed RNA is a sense RNA or an antisense RNA comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% sequence identity with or is identical to the coding or transcribed DNA sequence (sense) or to the complement (antisense) of part of the PARP gene whose expression is to be suppressed. Preferably, the transcribed DNA region does not code for a functional protein. Particularly, the transcribed region does not code for a protein. Further, the nucleotide sequence of the sense or antisense region should preferably be at least about 100 nucleotides in length, more preferably at least about 250 nucleotides, particularly at least about 500 nucleotides but may extend to the full length of the coding region of the gene whose expression is to be reduced.

For the purpose of this invention the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two

sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann ,1983) using a window-size of 20 nucleotides or amino acids, a word length of 2 amino acids, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can be conveniently performed using commercially available software packages such as the programs of the Intelligenetics™ Suite (Intelligenetics Inc., CA).

It will be clear to a skilled artisan that one or more sense or antisense PCD modulating chimeric genes can be used to achieve the goals of the first aspect of the invention. When one sense or antisense PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes. This can e.g. be achieved by choosing the transcribed region of the chimeric gene in such a way that expression of both classes of genes can be regulated by one sense or antisense RNA, i.e. by choosing target regions corresponding to the highest homology DNA region of the PARP genes of both classes and incorporating a sense or antisense transcribed DNA region corresponding to both target regions, conform to the conditions described above for sense and antisense RNA. Alternatively, different sense or antisense RNA regions, each specific for regulating the expression of one class of PARP genes, can be combined into one RNA molecule, encoded by one transcribed region of one PCD modulating chimeric gene. Obviously, the different sense or antisense RNA regions specific for regulating the expression of one class of PARP genes can be introduced as separate PCD modulating chimeric genes.

Preferred sense and antisense encoding transcribed regions comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the N-terminal domains of the PARP genes, preferably corresponding to a sequence of at least about 100 consecutive nucleotides selected from the sequence of SEQ ID No 1 from nucleotide position 113 to 1189, the sequence of SEQ ID No 3 from nucleotide position 107 to 583, the sequence of SEQ ID No 5 from nucleotide position 131 to 542 or the sequence of SEQ ID No 10 from nucleotide position 81 to 1180. However, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the N-terminal

domain of the PARP genes, or even to complete sequence of the PARP genes, particularly the protein-encoding region thereof. Further preferred are sense and antisense encoding transcribed regions which comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the C-terminal catalytic domains of the PARP genes, preferably a sequence of at least 100 nucleotides encompassing the PARP-signature encoding nucleotide sequences, particularly the PARP-signature encoding nucleotide sequences indicated *supra*. Again, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the C-terminal domain of the PARP genes.

In another particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of apparent activity of the endogenous PARPs of both classes. To this end, the transcribed DNA region encodes a biologically active RNA which is translated into a protein or peptide inhibiting NAP or ZAP class PARP proteins or both, such as inactivating antibodies or dominant negative PARP mutants.

"Inactivating antibodies of PARP proteins" are antibodies or parts thereof which specifically bind at least to some epitopes of PARP proteins, such as the epitope covering part of the ZN finger II from position 111-118 in ZAP1 or a corresponding peptide in ZAP2, and which inhibit the activity of the target protein.

"Dominant negative PARP mutants" as used herein, are proteins or peptides comprising at least part of a PARP protein (or a variant thereof), preferably a PARP protein endogenous to the eukaryotic target host cell, which have no PARP activity, and which have an inhibitory effect on the activity of the endogenous PARP proteins when expressed in that host cell. Preferred dominant negative PARP mutants are proteins comprising or consisting of a functional DNA binding domain (or a variant therof) without a catalytic domain (such as the N-terminal Zn-finger containing domain of about 355 to about 375 amino acids of a PARP of the ZAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 or a DNA binding protein domain comprising

the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11 from amino acid at position 1 to the amino acid at position 98, or such as the N-terminal DNA binding protein domain of about 135 to 160 amino acids of a PARP of the NAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138) or without a functional catalytic domain (such as inactive PARP mutants, mutated in the so-called PARP signature, particularly mutated at the conserved lysine of position 850 of SEQ ID No 2, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6). Preferably, dominant negative PARP mutants should retain their DNA binding activity. Dominant negative PARP mutants can be fused to a carrier protein, such as a β-glucuronidase (SEQ ID No 12).

Again, one or more PCD modulating genes encoding one or more dominant negative PARP mutants can be used to achieve the goals of the first aspect of the invention. When one PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes.

In another embodiment of the first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by modification of the nucleotide sequence of the endogenous PARP genes in those cells so that the encoded mutant PARP proteins retain about 10% of their activity. Methods to achieve such a modification of endogenous PARP genes include homologous recombination to exchange the endogenous PARP genes for mutant PARP genes e.g. by the methods described in US patent 5,527,695. In a preferred embodiment such site-directed modification of the nucleotide sequence of the endogenous PARP genes is achieved by introduction of chimeric DNA/RNA oligonucleotides as described in WO 96/22364 or US patent 5,565,350.

For plant cells, it has however been found that introduction of one PCD modulating chimeric gene, preferably encoding biologically active RNA active in reducing the expression of one class of the PARP genes, particularly of PARP genes of the ZAP class, may be sufficient for reduction of the total PARP activity in those plant cells in accordance with the first aspect of the invention, i.e. for inhibiting or preventing programmed cell death in those plant cells.

In this embodiment of the invention, the PCD modulating chimeric gene preferably comprises a transcribed region which codes for a biologically active RNA which comprises at least one RNA region, preferably of at least 100 nucleotides in length, classifying according to the herein mentioned criteria as a sense RNA for one of the endogenous PARP genes, and which comprises at least on other RNA region, preferably of at least 100 nucleotides in length, classifying according to the herein mentioned criteria as an antisense RNA for one of the endogenous PARP genes, whereby the antisense and sense RNA region are capable of combining into a double stranded region, preferably over a distance of at least about 100 nucleotides.

It is expected that introduction of one PCD modulating chimeric gene, which can decrease the functional or apparent level of one class of PARP proteins, particularly a PARP protein of the ZAP class, as herein described, may likewise be sufficient for reduction of the total PARP activity in plant cells in accordance with the first aspect of the invention.
The reduced or inhibited programmed cell death in plant cells comprising at least one PCD modulating chimeric gene in accordance with the first aspect of the invention can result in enhanced resistance to adversary conditions, such as resistance to stress imposed by treatment with chemicals, cold stress resistance, resistance to stress imposed by pathogens and pests , drought resistance, heat stress resistance etc.

In another aspect of the invention, programmed death of eukaryotic cells, preferably selected cells, particularly selected plant cells is enhanced by a severe decrease in the functional level of PARP, preferably reduced almost completely, such that the DNA repair and maintenance of the genome integrity are no longer possible.

In one embodiment of this aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced severely, particularly abolished almost completely, by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment of the second aspect of the invention, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is severely decreased, particularly almost completely eliminated.

As mentioned for the first aspect of this invention, the transcribed regions of the PCD modulating chimeric genes encode biologically active RNA, which can interfere with the expression of the endogenous PARP genes (e.g. through antisense action, co-suppression or ribozyme action) or the biologically active RNA can be further translated into a peptide or protein, capable of inhibiting the PARP proteins of the NAP and ZAP class, such as inactivating antibodies or dominant negative PARP mutants.

In a particularly preferred embodiment of the second aspect of the invention, the transcribed region of the PCD modulating chimeric genes (PCD enhancing chimeric genes) codes for a biologically active RNA which comprises at least one RNA region (preferably of at least about 100 nucleotides in length) classifying according to the above mentioned criteria as a sense RNA for at least one of the endogenous PARP genes, and at least one other RNA region (preferably of at least about 100 nucleotides in length), classifying according to the above mentioned criteria as an

antisense RNA for at least one of the endogenous PARP genes, whereby the antisense and sense RNA region are capable of combining into a double stranded RNA region (preferably over a distance of at least about 100 nucleotides). In an especially preferred embodiment, two such PCD modulating genes, one targeted to reduce the functional level of a PARP protein of the NAP class, and the other targeted to reduce the functional level of a PARP protein of the ZAP class are introduced into an eukaryotic cell or organism, preferably a plant cell or plant.

It is clear that the different embodiments for the transcribed DNA regions of the chimeric PCD modulating genes of the invention can be used in various combinations to arrive at the goals of the invention. E.g. a first chimeric PCD modulating gene may encode a sense RNA designed to reduce the expression of an endogenous PARP gene of the ZAP class, while the second chimeric PCD modulating gene may encode a dominant negative PARP mutant designed to reduce the expression of an endogenous PARP gene of the NAP class.

Whether the introduction of PCD modulating chimeric genes into eukaryotic cells will ultimately result in a moderately reduced or a severally reduced functional level of combined PARP in those cells -i.e. in inhibited PCD or enhanced PCD- will usually be determined by the expression level (either on transcriptional level or combined transcriptional/tranlational level) of those PCD modulating genes. A major contributing factor to the expression level of the PCD modulating gene is the choice of the promoter region, although other factors (such as, but not limited to, the choice of the 3'end, the presence of introns, codon usage of the transcribed region, mRNA stability, presence of consensus sequence around translation initiation site, choice of 5' and 3' untranslated RNA regions, presence of PEST sequences, the influence of chromatin structure surrounding the insertion site of a stabile integrated PCD modulating gene, copy number of the introduced PCD modulating genes, etc.) or combinations thereof will also contribute to the ultimate expression level of the PCD modulating gene. In general, it can be assumed that moderate reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively weak promoter, while severe reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively strong promoter. However, the expression level of a PCD modulating gene comprising a

specific promoter and eventually its effect on PCD, can vary as a function of the other contributing factors, as already mentioned.

For the purpose of particular embodiments of the invention, the PCD modulating chimeric genes may comprise a constitutive promoter, or a promoter which is expressed in all or the majority of the cell types throughout the organism, particularly throughout the plant, such as the promoter regions derived from the T-DNA genes, particularly the opine synthase genes of *Agrobacterium* Ti- or Ri-plasmids (e.g. nos, ocs promoters), or the promoter regions of viral genes (such as CaMV35S promoters, or variants thereof).

It may be further be advantageous to control the expression of the PCD modulating gene at will or in response to environmental cues, e.g. by inclusion of an inducible promoter which can be activated by an external stimuli, such as, but not limited to application of chemical compounds (e.g. safeners, herbicides, glucocorticoids), light conditions, exposure to abiotic stress (e.g. wounding, heavy metals, extreme temperatures, salinity or drought) or biotic stress (e.g. pathogen or pest infection including infection by fungi, viruses, bacteria, insects, nematodes, mycoplasms and mycoplasma like organisms etc.). Examples of plant-expressible inducible promoters suitable for the invention are: nematode inducible promoters (such as disclosed in WO 92/21757), fungus inducible promoters (WO 93/19188, WO 96/28561), promoters inducible after application of glucocorticoids such as dexamethasone ( ), or promoters repressed or activated after application of tetracyclin (Gatz *et al.* 1988 ; Weimann *et al.* 1994)

In several embodiments of the invention, particularly for the second aspect of the invention (i.e. enhanced PCD), it may be convenient or required to restrict the effect on programmed cell death to a particular subset of the cells of the organism, particularly of the plant, hence the PCD modulating genes may include tissue-specific or cell type-specific promoters. Examples of suitable plant-expressible promoters selectively expressed in particular tissues or cell types are well known in the art and include but are not limited to seed-specific promoters (e.g. WO89/03887), organ-primordia specific promoters (An *et al.*, 1996), stem-specific promoters (Keller *et al.*, 1988), leaf specific promoters (Hudspeth *et al.* ,1989), mesophyl-specific

promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller *et al.*,1989), tuber-specific promoters (Keil *et al.*, 1989), vascular tissue specific promoters (Peleman *et al.*, 1989), meristem specific promoters (such as the promoter of the *SHOOTMERISTEMLESS* (*STM*) gene, Long *et al.*, 1996), primordia specific promoter (such as the promoter of the *Antirrhinum* CycD3a gene, Doonan *et al.*, 1998), anther specific promoters (WO 89/10396, WO9213956, WO9213957) stigma-specific promoters (WO 91/02068), dehiscence-zone specific promoters (WO 97/13865), seed-specific promoters (WO 89/03887) etc.

Preferably the chimeric PCD modulating genes of the invention are accompanied by a marker gene, preferably a chimeric marker gene comprising a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable "color" to the transformed plant cell, such as the A1 gene (Meyer *et al.*, 1987) or Green Fluorescent Protein (Sheen *et al.*, 1995), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provided antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

Methods to introduce PCD modulating chimeric genes into eukaryotic cells, particularly methods to transform plant cells are well known in the art, and are believed not to be critical for the methods of the invention. Transformation results in either transient or stably transformed cells (whereby the PCD modulating chimeric genes are stably inserted in the genome of the cell, particularly in the nuclear genome of the cell).

It is clear that the methods and means described in this invention to alter the programmed cell death in eukaryotic cells and organisms, particularly in plant cells and plants, has several important application possibilities. Inhibition of PCD by the

methods and means of the invention, can be used to relieve the stress imposed upon the cells, particularly the plant cells, during transformation and thus to increase transformation efficiency, as described in WO 97/06267. Inhibition of PCD can also be used to improve cell culture of eukaryotic cells, particularly of plant cells. Triggering of PCD in particular cell types using the means and methods of the invention, can be used for methods which call upon the use of a cytotoxin. Since PCD is the "natural" way for cells to die, the use of PCD enhancing chimeric genes of the invention constitutes an improvement over the use of other cytotoxic genes such as RNAse or diptheria toxin genes which lead to cell lysis. Moreover, low-level expression of PCD enhancing genes in cells different than the targeted cells, will lead to a moderate reduction instead of a severe reduction of PARP activity in those cells, thus actually inhibiting PCD in non-target cells.

For plants, preferred applications of PCD enhancing chimeric genes include, but are not limited to:

1. the generation of plants protected against fungus infection, whereby the PCD enhancing chimeric gene or genes comprise a fungus-responsive promoter as described in WO 93/19188 or WO 96/28561.
2. the generation of nematode resistant plants, whereby the PCD enhancing chimeric gene or genes comprise a nematode inducible promoters such as disclosed in WO 92/21757
3. the generation of male or female sterile plants, whereby the PCD enhancing chimeric gene or genes comprise anther-specific promoters (such as disclosed in WO 89/10396, WO9213956, WO9213957) or stigma-specific promoters (such as disclosed in WO 91/02068)
4. the generation of plants with improved seed shatter characteristics whereby the PCD enhancing chimeric gene or genes comprise dehiscence zone-specific promoters (such as disclosed in WO 97/13865).

Unexpectedly, it has been found that upon introduction of a PCD modulating chimeric gene according to the first aspect of the invention, preferably a chimeric gene modulating the expression of a PARP gene of the ZAP class, particularly a chimeric gene modulating the expression of a PARP gene of the ZAP class wherein the transcribed region codes for a biologically active RNA comprising simultaneously

a sense and antisense RNA as herein described, the transformed plant cells, plant calli and plants exhibited an enhanced growth.

Although not intending to limit the invention to a particular mode of action, it is believed that the enhanced growth is a consequence of the reduced number of cells which undergo programmed cell death, probably by increasing the threshold for a signal inhibiting cell division, thus leading to more vigorously growing plants. These plants are also more stress resistant as explained elsewhere in this application.

Therefore, in a third aspect, the invention also relates to a method for enhancing growth, preferably vegetative growth, of plant cells, plant tissues and plants comprising at least one PCD modulating chimeric gene according the first aspect of the invention preferably a chimeric gene modulating the expression of a PARP gene of the ZAP class, particularly a chimeric gene modulating the expression of a PARP gene of the ZAP class wherein the transcribed region codes for a biologically active RNA comprising simultaneously a sense and antisense RNA.

Although it is clear that the invention can be applied essentially to all plant species and varieties, the invention will be especially suited to alter programmed cell death in plants with a commercial value. Particularly preferred plants to which the invention can be applied are corn, oil seed rape, linseed, wheat, grasses, alfalfa, legumes, a brassica vegetable, tomato, lettuce, cotton, rice, barley, potato, tobacco, sugar beet, sunflower, and ornamental plants such as carnation, chrysanthemum, roses, tulips and the like.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric cell-division controlling gene of the invention in other varieties of the same or related plant species. Seeds obtained from the transformed plants contain the PCD modulating gene of the invention as a stable genomic insert.

The following non-limiting Examples describe the construction of chimeric apoptosis controlling genes and the use of such genes for the modulation of the programmed cell death in eukaryotic cells and organisms. Unless stated otherwise in the

Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1: DNA sequence of the ZAP gene of *Zea mays* (*zap1*)
SEQ ID No 2: protein sequence of the ZAP protein of *Zea mays* (ZAP1)
SEQ ID No 3: DNA sequence of the NAP gene of *Zea mays* (*nap*)
SEQ ID No 4: protein sequence of the NAP protein of *Zea mays* (NAP)
SEQ ID No 5: DNA sequence of the NAP gene of *Arabidopsis thaliana* (*app*)
SEQ ID No 6: protein sequence of the NAP protein of *Arabidopsis thaliana* (APP)
SEQ ID No 7: consensus sequence for the A domain of non-conventional PARP proteins
SEQ ID No 8: consensus sequence for the A1 domain of non-conventional PARP proteins
SEQ ID No 9: consensus sequence for the A2 domain of non-conventional PARP proteins
SEQ ID No 10: DNA sequence of the second ZAP gene of *Zea mays (Zap2)*
SEQ ID No 11: protein sequence of the ZAP protein of *Zea mays* (ZAP2)
SEQ ID No 12: amino acid sequence of a fusion protein between the DNA binding domain of APP and the GUS protein
SEQ ID No 13: degenerated PCR primer
SEQ ID No 14: degenerated PCR primer
SEQ ID No 15: PCR primer
SEQ ID No 16: PCR primer
SEQ ID No 17: PCR primer
SEQ ID No 18: PCR primer
SEQ ID No 19: PCR primer

SEQ ID No 20: PCR primer

SEQ ID No 21: app promoter-gus translational fusion

Sequence listing free text

The following free text has been used in the Sequence Listing part of this application

<223> Description of Artificial Sequence:A domain of

"

non-conventional PARP proteins

<223> Description of Artificial Sequence:A1 domain on

non conventional PARP protein

<223> Description of Artificial Sequence: A2 domain of

"

non-conventional PARP protein

"

<223> Description of Artificial Sequence: fusion protein

between APP N-terminal domain and GUS protein

<223> Description of Artificial Sequence: degenerated

"

PCR primer

<223> Description of Artificial Sequence:oligonucleotide

"

for use as PCR primer

"

<223> Description of Artificial Sequence: APP promoter

fusion with beta-glucuronidase gene

<223> translation initiation codon

Examples

Experimental procedures

Yeast and bacterial strains

Saccharomyces cerevisiae strain DY (MATa *his3 can1-10 ade2 leu2 trp1 ura3::*(3xSV40 AP1-lacZ) (Kuge and Jones, 1994) was used for the expression of the APP protein. Yeast transformation was carried out according to Dohmen *et al.* (1991). Strains were grown on a minimal SDC medium (0.67% yeast nitrogen base, 0.37% casamino acids, 2% glucose, 50 mg l^{-1} of adenine and 40 mg l^{-1} of tryptophan). For the induction of the APP expression, glucose in SDC was substituted with 2% galactose.

Escherichia coli strain XL-I (Stratagene, La Jolla, CA) was used for the plasmid manipulations and library screenings, which were carried out according to standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). *E. coli* BL21 (Studier and Moffat, 1986) was used for the APP protein expression and *Agrobacterium tumefaciens* C58C1Rif^R(pGV2260) (Deblaere *et al.*, 1985) for the stable transformation of plants.

Poly(ADP-ribose)polymerase activity assay

Enzymatic activity of the APP was assayed in total protein extracts of yeast strains prepared as follows. DY(pV8SPA) or DY(pYeDP1/8-2) were grown in 50 ml of SDC medium overnight at 30°C on a gyratory shaker at 150 rpm. Yeast cells were harvested by centrifugation at 1,000×*g*, washed three times with 150 ml of 0.1 M potassium phosphate buffer (pH 6.5), and resuspended in 5 ml of sorbitol buffer (1.2 M sorbitol, 0.12 M K_2HPO_4, 0.033 M citric acid, pH 5.9). Lyticase (Boehringer, Mannheim, Germany) was added to the cell suspension to a final concentration of 30 U ml^{-1} and cells were incubated at 30°C for 1 h. Yeast spheroplasts were then washed three times with sorbitol buffer and resuspended in 2 ml of ice-cold lysis buffer (100 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT). After sonication, the lysate was centrifuged at 20,000×*g* for 20 min at 4°C and the

supernatant was desalted on a Econo-Pack™ 10 DG column (Bio-Rad, Richmond, CA) equilibrated with reaction buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT). To reduce proteolytic degradation of proteins, the lysis and reaction buffers were supplemented with a protease inhibitor cocktail (Boehringer), one tablet per 50 ml. Nucleic acids were removed from the total extracts by adding NaCl and protamine sulfate to a final concentration of 600 mM and 10 mg ml^{-1}, respectively. After incubation at room temperature for 10 min, the precipitate was removed by centrifugation at 20,000×*g* for 15 min at 4°C. The buffer of the supernatant was exchanged for the reaction buffer by gel filtration on an Econo-Pack™ 10 DG column.

The assay for the synthesis of poly(ADP-ribose) was adapted from Collinge and Althaus (1994). Approximately 500 µg of total yeast protein were incubated in a reaction buffer supplemented with 30 µCi of ^{32}P-NAD^+ (500 Ci $mmol^{-1}$), unlabeled NAD^+ to a final concentration of 60 µM, and 10 µg ml^{-1} sonicated salmon sperm DNA. After incubation for 40 min at room temperature, 500 µl of the stop buffer (200 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 5 mM EDTA, 1% Na^+-N-lauroyl-sarcosine, and 20 µg ml^{-1} proteinase K) were added and reactions incubated at 37°C overnight. After phenol and phenol/chloroform extractions, polymers were precipitated with 2.5 volumes of ethanol with 0.1 M NaAc (pH 5.2). The pellet was washed with 70% ethanol, dried, and dissolved in 70% formamide, 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol. Samples were heated at 80°C for 10 min and then loaded onto a 12% polyacrylamide/6 M urea sequencing gel. Gels were dried on 3MM paper (Whatman International, Maidstone, UK) and exposed either to Kodak X-Omat X-ray film (Eastman Kodak, Richmond, NY) or scanned using a PhosphorImager™ 445SI (Molecular Dynamics, Sunnyvale, CA).

Immunological techniques

A truncated *app* cDNA encoding an APP polypeptide from amino acids Met^{310} to His^{637} was expressed as a translation fusion with six histidine residues at the N terminus after induction of a 500-ml culture of the *E. coli* BL21(pETΔNdeSPA) with 1 mM isopropyl-ß-D-thiogalactopyranoside. The APP polypeptide was purified to near homogeneity by affinity chromatography under denaturing conditions (in the presence

of 6 M guanidinium hydrochloride) on a Ni^{2+}-NTA-agarose column, according to the manufacturer's protocol (Qiagen, Chatsworth, CA). After dialysis against PBS, a mixture of the soluble and insoluble APP polypeptides was used to immunize two New Zealand White rabbits following a standard immunization protocol (Harlow and Lane, 1988). For the Western blot analysis, proteins were resolved by denaturing SDS-PAGE (Sambrook *et al.*, 1989; Harlow and Lane, 1988) and transferred onto nitrocellulose membranes (Hybond-C; Amersham), using a Semi-Dry Blotter II (Kem-En-Tec, Copenhagen, Denmark).

In situ antigen localization in yeast cells was carried out as described (Harlow and Lane, 1988). For the localization of the APP protein in yeast spheroplasts, anti-APP serum was diluted 1:3,000 to 1:5,000 in Tris-buffered saline-BSA buffer. 10H monoclonal antibody, which specifically recognizes poly(ADP-ribose) polymer (Ikajima *et al.*, 1990) was used in a 1:100 dilution in PBS buffer. The mouse antibody were detected with the sheep anti-mouse IgG $F(ab')_2$ fragment conjugated to fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:200. Rabbit IgG was detected with CY-3 conjugated sheep anti-rabbit IgG sheep $F(ab')_2$ fragment (Sigma), at a dilution of 1:200. For the visualization of DNA, slides were incubated for 1 min in PBS with 10 µg ml^{-1} of 4',6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence imaging was performed on an Axioskop epifluorescence microscope (Zeiss, Jena, Germany). For observation of FITC and CY-3 fluorochromes, 23 and 15 filter cubes were used, respectively. Cells were photographed with Fuji Color-100 super plus film.

Plant material and histochemical analysis

Nicotiana tabacum SR1 (Maliga *et al.*, 1975) was used for the generation of stable transformants following the procedure of leaf disc cocultivation (De Block *et al.*, 1987) with *A. tumefaciens* C58C1Rif^R(pGV2260; pGCNSPAGUS). *N. tabacum* SR1 line transformed with authentic GUS under the control of the 35S CaMV was used as a control. *Arabidopsis thaliana* ecotype Columbia was used for the transformation of the *app*-promoter-GUS fusion following the *in situ* infiltration procedure.

For *in situ* histochemical staining of the GUS activity, plant samples were fixed in ice-cold 90% acetone for 30 min, washed in 0.1 M K_2HPO_4 (pH 7.8), and then

incubated in staining buffer (0.1 M K_2HPO_4, pH 7.8, 2 mM X-Gluc, 20 mM Fe^{3+}-EDTA) at 37°C. Stained plant tissues were stored in 70% ethanol at 4°C. When necessary, browning of tissues due to phenolic oxidation was reduced by incubation with lactophenol (Beeckman and Engler, 1994). The GUS staining was examined under a Jenalumar light microscope (Zeiss). Plant tissues were photographed with Fuji Color-100 super plus film.

Miscellaneous methods

The plasmid construction steps were routinely verified by DNA sequencing carried out according to protocols provided by USB Biochemicals (Cleveland, OH). ^{32}P-labeled DNA probes for nucleic acid hybridization were synthesized by the Ready-Prime DNA labelling kit (Amersham). For DNA and RNA hybridization experiments, the buffer system of Church and Gilbert (1984) was used (0.25 M sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA). For Western blot analysis, yeast total proteins were extracted with phenol essentially as described for plant tissues (Hurkman and Tanaka, 1986). For Northern blot analysis, total yeast RNA was extracted with hot phenol as described (Ausubel *et al.*, 1987). RNA was resolved on 1.5% agarose gels after denaturation with glyoxal (Sambrook *et al.*, 1989). Hybond-N nylon filters (Amersham) were used for the nucleic acid blotting.

Example 1: Isolation of genes encoding PARP homologues from *Zea mays*.

With the purpose of isolating maize cDNA encoding PARP homologue(s) two approaches were followed. First, a maize cDNA library was screened under low-stringency DNA—DNA hybridization conditions using a DNA probe prepared from the *Arabidopsis app* cDNA. Secondly, PCR amplification of part of the maize PARP was performed, using the first-strand cDNA as a template and two degenerate primers, designed on the basis of the sequence of the "PARP signature", the most conserved amino acid sequence between all known PARP proteins.

A λZAP (Stratagene) cDNA library from leaves of maize (*Zea mays* L.), inbred line B734 . Plaques (500,000) were screened according to standard procedures (Sambrook *et al.*. 1989). After screening with the *Arabidopsis app* probe, one

non-full-length cDNA of 1.4 kbp was purified. After the initial cDNA library screening with the *app* probe and a subsequent 5' rapid amplification of cDNA ends (RACE) PCR analysis, the *nap* gene, a maize homologue of the *Arabidopsis app*, was identified. For the 5'RACE PCR, the template was prepared with the Marathon kit (Clontech, Palo Alto, CA) and 0.5 µg of maize poly(A)$^+$ RNA isolated from inner sheath, outer sheath, and leaves of 1-week-old maize seedlings. The gene-specific, nested primers for PCR amplification were 5'-GGGACCATGTAGTTTATCTTGACCT-3' (SEQ ID No 15) and 5'-GACCTCGTACCCCAACTCTTCCCCAT-3' (SEQ ID No 16) for *nap* primers. The amplified PCR products were subcloned and sequenced. A fragment of 800 bp was amplified with *nap*-specific primers which allowed to reconstruct the 2295-bp-long sequence of *nap* cDNA (SEQ ID No 3).

The NAP protein was 653 amino acids long (molecular mass ~73 kDa; SEQ ID No 4) and highly similar (61% sequence identity and 69% similarity) to the APP. Most importantly, NAP had an organization of the N-terminus congruent to APP (Figure 1A), suggesting a rather strict selection pressure on the structure of APP-like proteins in plants. The *nap* gene was unique in the maize genome (Figure 2A) and encoded a transcript of 2.4 kb (Figure 2C).

Using degenerate primers based on very highly conserved regions in the "PARP signature" and first-strand cDNA from *Zea mays* as a template, a 310-bp fragment was amplified. For the PCR with degenerate primers 5'-CCGAATTCGGNTAYATGTTYGGNAA-3' (SEQ ID No 13) and 5'-CCGAATTCACNATRTAYTCRTTRTA-3' (SEQ ID No 14) with Y=C/T; R=A/G; N=A/G/C/T), the first strand cDNA was used as a template and was synthesized using 5 µg of poly(A)$^+$ RNA from young maize leaves and MuMLV reverse transcriptase. PCR amplifications were performed with *Taq* DNA polymerase in 100 µl volume using the following conditions: 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, followed by 38 cycles of 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, with a final incubation for 10 min at 72°C.

The sequence of the 310 bp fragment showed 55% sequence identity and 64% sequence similarity with human PARP over the same region, but was, however, different from the sequence of the *nap* cDNA. Three *zap* cDNAs were identified after screening with the 310-bp fragment, which was obtained by PCR with degenerate primers. These three purified cDNA were all derived from the same transcript because they had identical 3' non-coding regions; the longest clone (#9) was sequenced on both strands (SEQ ID No 1). This cDNA encoded a PARP-homologous polypeptide of 689 amino acids (SEQ ID No 2; molecular mass ~109 kDa), which we designated as ZAP1 (Figure 1B). The first Zn-finger of ZAP1 was probably nonfunctional because it had the sequence CKSCxxxHASV, which included no third cysteine residue.

5'RACE PCR analysis of *zap* transcripts from the maize line LG2080 (the screened cDNA library was made from the inbred line B734) was performed as described above using the following *zap* specific primers 5'-AAGTCGACGCGGCCGCCACACCTAGTGCCAGGTCAG-3' (SEQ ID No 17) and 5'-ATCTCAATTGTACATTTCTCAGGA-3' (SEQ ID No 18). A 450-bp PCR product was obtained after PCR with *zap*-specific primers. Eight independent, because of their slight differences in lengths at their 5' ends, 5'RACE PCR fragments generated with *zap*-specific primers were sequenced. In all the transcripts from the LG2080 maize plants, there was an insertion of additional sequence in the coding region, which made the ZAP protein longer by 11 amino acids (980 amino acids, molecular mass ~110.4 kDa). The Zn-finger I of ZAP2 was standard and read CKSCxxxHARC (Figure 1B; SEQ ID No 11). The sequence difference may be due either to differences between maize varieties, to the expression of two homologous genes, or to alternative splicing. In fact, maize may have at least two *zap* genes (Figure 2B), which encode a transcript of 3.4-3.5 kb (Figure 2D). The DNA gel blot experiment with a probe prepared from the *zap* cDNA showed that homologous genes were present in *Arabidopsis*.

Structurally ZAP was very similar to PARP from animals. It had a well conserved DNA-binding domain composed of two Zn-fingers (36% identity and 45% similarity to the DNA-binding domain of mouse PARP). Even higher homology was shown by comparing only the sequences of the Zn-fingers, Ala^{1}-Phe^{182} in the mouse enzyme (44% identity and 54% similarity), or a subdomain downstream from the nuclear

localization signal (NLS), Leu^{237}-Ser^{360} in mouse PARP (40% identity and 50% similarity). Whereas the bipartite nuclear localization signal characteristic of mammalian PARP could not be identified in ZAP, the sequence KRKK fitted a monopartite NLS (Figure 1B). The putative automodification domain was poorly conserved and was shorter in ZAP than in mouse PARP. The compilation of the homology of the catalytic dmains between ZAP, NAP, APP and mouse PARP is shown in Figure 2. It should be noted that the NAD^+-binding domain of ZAP was more similar to the mammalian enzyme (48% identity) than to that of APP and NAP (40% and 42% sequence identity, respectively), whereas APP and NAP were 68% identical and 76% similar in their catalytic domain.

Example 2 Demonstration that non-conventional PARP protein has a DNA-dependent poly(ADP-ribose) polymerase activity.

APP is a DNA-dependent poly(ADP-ribose) polymerase

A more detailed study of the APP protein (expressed in yeast) was performed to understand the activity of PARP-like proteins from the NAP class. The choice of yeast as the organism for the expression and enzymatic analysis of the *Arabidopsis* APP protein was made for a number of reasons. As an eukaryote, *Saccharomyces cerevisiae* is better suited for the expression of native proteins from other eukaryotic organisms, and unlike most other eukaryotic cells, it does not possess endogenous PARP activity (Lindahl *et al.*, 1995).

The full-length *app* cDNA was placed in pYeDP1/8-2 under the control of a galactose-inducible yeast promoter in the following way. the full-length *app* cDNA was excised from pC3 (Lepiniec *et al.*, 1995) as an *Xho*I-*Eco*RI fragment. The ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was subcloned into the *Sma*I site of the yeast expression vector pYeDP1/8-2 (Cullin and Pompon, 1988). The resulting expression vector pV8SPA (Figure 4A) was transformed into *S. cerevisiae* strain DY.

For APP expression in *E. coli*, the complete coding region of the *app* cDNA was PCR amplified with *Pfu* DNA polymerase (Stratagene), using the primers

5'-AGGATCCCATGGCGAACAAGCTCAAAGTGAC-3' (SEQ ID No 19) and 5'-AGGATCCTTAGTGCTTGTAGTTGAAT-3' (SEQ ID No 20), and subcloned as a *BamH*I fragment into pET19b (Novagene, Madison, WI), resulting in pETSPA. The expression of the full-length APP in *E. coli* BL21 from pETSPA was very poor. To obtain better expression, pETSPA was digested with *Nco*I and *Nde*I or with *Sma*I, the ends were filled in by the Klenow fragment of DNA polymerase I, and the plasmids were then self-ligated. Of the resulting plasmids pETΔNdeSPA and pETΔSmaSPA, only pETΔNdeSPA gave satisfactory expression of the truncated APP polypeptide (Met^{310} to His^{637}) in *E. coli* BL21.

The expression of the APP in yeast was verified by Northern and Western blot analysis. (Fig 4) As the promoter in pV8SPA is inactive when cells are grown on glucose and derepressed on galactose-containing media, the expression was expected to be tightly regulated by the carbon source. However, Northern blot analysis of RNA and immunoblot analysis of proteins in DY(pV8SPA) as compared to the control DY strain containing the empty vector, showed that app mRNA and APP protein were expressed in yeast even when grown on glucose-containing media (Figure 4B, lane 2). The peculiarity of the expression observed on glucose-containing medium was that both app mRNA and APP protein were shorter than the ones detected after induction with galactose (compare lanes 2 and 4 in Figure 4B). The APP polypeptide with the higher molecular weight, (apparently a full-length protein) was only detected on galactose-containing medium, although such cells also expressed the truncated mRNA and protein. The most probable explanation for this finding is that when the DY(pV8SPA) strain is grown on glucose, there is a leaky expression from the expression cassette, with transcription beginning 200-300 bp downstream from the transcription start observed after galactose induction. This shorter mRNA probably does not code for the first methionine (Met^{1}) of APP and, therefore, translation is initiated at Met^{72}. This would explain the observed difference of -5 kDa (calculated difference being 7.5 kDa) in the molecular masses of the APP polypeptides from strains grown on glucose or on galactose. The possibility that the differences in molecular masses may be attributed to self-modification through poly(ADP-ribos)ylation was ruled out by growing strains in the presence of PARP

inhibitors, such as 3ABA and nicotinamide (Figure 4B, compare lanes 6 and 8 to lane 4).

To detect the synthesis of poly(ADP-ribose), total proteins were extracted from yeast strains grown under different conditions and incubated in the presence of radioactively labeled NAD^+. To prevent synthesis of poly(ADP-ribose) and possible automodification of the APP *in vivo*, strains were also grown in the presence of 3ABA, a reversible inhibitor of PARP, which was subsequently removed from the protein extracts during desalting. Figure 5 shows that poly(ADP-ribose) is synthesized by protein extracts of DY(pV8SPA) grown on galactose (Figure 5A, lanes 1 and 2), but not by a strain containing the empty vector (Figure 5A, lane 4). It can also be seen that *Arabidopsis* APP could synthesize polymers up to 40 residues in length (Figure 5A, lane 1) with the majority of the radioactivity being incorporated into 10-15-mer. This observation is consistent with the polymer sizes detected by other authors (Chen *et al.*, 1994). More radioactivity was incorporated into polymer when the yeast strain was grown with 3ABA than without (Figure 5A, lane 1 compared to lane 2); the reason might be that either the APP extracted from inhibited cultures was less automodified (it is believed that automodification inhibits the activity of PARP) or the labeled NAD^+ was used by the enzyme from the uninhibited culture for the extension of existing polymer, resulting in a lower specific activity overall. Under the same reaction conditions poly(ADP-ribose) synthesized by human PARP, either in reaction buffer alone or in the presence of a yeast total protein extract from DY(pYeDP1/8-2) (Figure 5A, lanes 5 and 6, respectively), showed much longer chains, possibly up to 400-mer (de Murcia and Ménissier de Murcia, 1994).

The stimulation of enzymatic activity by nicked DNA is a well known property of PARP from animals (Alvarez-Gonzalez and Althaus, 1989). We therefore tested whether the activity of the APP protein was DNA dependent. After removal of yeast nucleic acids (DNA, RNA) and some basic proteins from the galactose-grown DY(pV8SPA) protein extract the synthesis of poly(ADP-ribose) was analyzed in the presence of increasing concentrations of sonicated salmon sperm DNA. As can be seen in Figure 5B, there was a direct correlation between the amount of DNA present in the reaction and the incorporation of ^{32}P-NAD^+. Scanning of the phosphor-images indicated that ~6-fold more radioactivity was incorporated into poly(ADP-ribose) in the reaction mixture

containing 40 μg ml^{-1} of DNA than into that with 2 μg ml^{-1} of DNA (Figure 5B, lanes 4 and 2, respectively). The synthesis of the polymer was sensitive to 3ABA in the reaction mix (Figure 5B, lane 5).

APP is a nuclear protein

In animal cells PARP activity is localized in the nucleus (Schreiber *et al.*, 1992). The intracellular localization, if nuclear, of APP could provide an important additional indication that APP is a *bona fide* plant PARP. To this end, the localization of the APP polypeptides in yeast cells was analyzed using anti-APP antisera. The APP polypeptide synthesized in yeast grown on galactose was found mainly in the nucleus. This localization was unaffected by the presence in the media of the PARP inhibitors.

In addition, we tested whether APP was constitutively active in yeast cells, as has been reported for the human PARP (Collinge and Althaus, 1994). Here, fixed yeast spheroplasts were incubated with monoclonal 10H antibody, which specifically recognizes poly(ADP-ribose) polymers (Kawamitsu *et al.*, 1984). A positive yellowish-green fluorescence signal with 10H antibody was localized in the nucleus and was observed only in DY(pV8SPA) cells grown on galactose. Positive staining was greatly reduced in cells grown in the presence of the PARP inhibitors, 3ABA and nicotinamide.

To identify the intracellular localization of APP in plant cells, a widely adopted approach in plant studies was used, *i.e.*, the examination of the subcellular location of a fusion protein formed between the protein in question and a reporter gene, once the protein fusion was produced in transgenic plants or transfected cells (Citovsky *et al.*, 1994; Sakamoto and Nagatani, 1996; Terzaghi *et al.*, 1997; von Arnim and Deng, 1994). An N-terminal translational fusion of GUS with the part of the APP polypeptide extending from the Met^{1} to Pro^{407} was made. The translational fusion of APP with bacterial GUS was constructed as follows. Plasmid pETSPA was cut with *Sma*I, treated with alkaline phosphatase, and ligated to a blunted *Nco*I-*Xba*I fragment from pGUS1 (Plant Genetic Systems N.V., Gent, Belgium). The ligation mix was transformed into *E. coli* XL-I and cells were plated onto LB medium supplemented with 0.1 mM isopropyl-ß-D-thiogalactopyranoside, 40 μg ml^{-1}

5-bromo-4-chloro-3-indolyl-ß-D-glucuronide, and 100 µg ml^{-1} of ampicillin. In this way, pETSPAGUS was selected as blue colonies. The expression in *E. coli* of the ~110-kDa fusion protein was confirmed by *in situ* GUS activity gels (Lee *et al.*, 1995). The APP-GUS fusion was placed under the control of the 35S promoter of the CaMV (the Klenow-blunted *Bam*HI fragment from pETSPAGUS was subcloned into *Sma*I-digested pJD330; Gallie and Walbot, 1992) and the resulting expression cassette was subcloned as an *Xba*I fragment into the *Xba*I site of the pCGN1547 binary vector (McBride and Summerfelt, 1990) to give pGCNSPAGUS. The pGCNSPAGUS was finally introduced into *A. tumefaciens* C58C1Rif^R(pGV2260) by the freezing-thawing transformation procedure.

Expression of the fusion protein was verified in *E. coli*. The chimeric cDNA under the control of the 35S CaMV promoter was stably integrated into the tobacco genome. Progeny from four independent transgenic tobacco plants were analyzed for the subcellular distribution of the GUS activity after *in situ* histochemical staining (Jefferson *et al.*, 1987). In 2-day-old seedlings GUS activity could be detected in cotyledons and in roots, but not in hypocotyls or root tips. Because of the transparency of root tissues, GUS staining was clearly localized in the nuclei of root hairs and epidermal cells. Additionally, some diffuse, non-localized staining of other root cells was seen, in particular along the vascular cylinders. This non-nuclear GUS staining was more pronounced in leaf tissues. Whereas young true leaves or cotyledons displayed intense blue staining of the nuclei, there was also some diffuse staining of the cytoplasm. In fully expanded leaves, however, GUS staining became homogenous and similar to the staining of control plants transformed with GUS under the control of the CaMV 35S promoter, in which GUS was expressed in the cytoplasm. Eventually, older leaves or cotyledons exhibited practically no histochemically detectable GUS activity, with the exception of the vascular bundles, where the GUS staining could not be confined to any particular cell compartment.

Deficiency in DNA ligase I induces expression of the app *gene*

PARP in animal cells is one of the most abundant nuclear proteins and its activity is regulated by allosteric changes in the protein upon binding to damaged DNA. We found that the *app* gene in *Arabidopsis* had a rather low level of expression,

suggesting that transcriptional activation of this gene might be essential for APP function *in vivo*. To test this hypothesis, the expression of the *app* gene was studied during *in vivo* genome destabilization caused by a DNA ligase I deficiency. A T-DNA insertion mutation, line SK1B2, in the *Arabidopsis* DNA ligase I gene was isolated previously (Babiychuk *et al.*, 1997). The mutation is lethal in the homozygous state, but the mutant allele shows normal transmission through the gametes. We therefore expected that cells homozygous for the mutation would die due to incomplete DNA synthesis during the S phase of the cell cycle, soon after the fertilization of the mutant embryo sac with mutant pollen.

An *app* promoter-GUS translational fusion , in which the coding region of GUS was fused in-frame with the first five amino acids of APP and 2 kb of *app* 5' flanking sequences was constructed (SEQ ID No 21). The gene encoding the fusion protein was transformed into *Arabidopsis*. After two back-crosses to a wild type, heterozygous plants transformed with *app* promoter-GUS were crossed with *Arabidopsis* line SK1B2. The inflorescences of the control plants and plants heterozygous for the ligase mutation were stained for the activity of GUS. The GUS staining pattern mostly detected in aging tissues probably reflects the expression of the *app* gene, although we have no firm evidence that all of the regulatory sequences were present in the constructs used. This pattern was the same both in the inflorescences of control plants, not carrying the mutant ligase gene and plants heterozygous for a mutation. Approximately one-fourth of the ovules in the mutant plants with the fusion protein are GUS positive. Closer microscopical examination showed that in the GUS-positive ovules only the gametophyte was stained. The only difference between the control plants and the mutant plant was a mutation in a DNA ligase gene. We therefore conclude that the *app* gene is induced because of either the accumulation of DNA breaks, or the death of the mutant embryo sacs fertilized with mutant pollen. GUS staining of embryo sacs was found to appear within 24 h after pollination, or therefore very soon after fertilization.

Example 3. Construction of PCD modulating chimeric genes and introduction of the T-DNA vectors comprising such PCD modulating genes in an *Agrobacterium* strain.

3.1. Construction of the p35S:(dsRNA-APP) and p35S:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 1 and 5):
For the p35S:(dsRNA-ZAP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

For the p35S:(dsRNA-APP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

3.2. Construction of the pNOS:(dsRNA-APP) and pNOS:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 2 and 6):
For the pNOS:(dsRNA-ZAP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

For the pNOS:(dsRNA-APP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

3.3. Construction of the pTA29:(dsRNA-APP) and pTA29:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 3 and 7):
For the pTA29:(dsRNA-ZAP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

For the pTA29:(dsRNA-APP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

3.4. Construction of the pNTP303:(dsRNA-APP) and pNTP303:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 4 and 8):
For the pNTP303:(dsRNA-ZAP) chimeric gene

- a NTP303 promoter region (Wetering 1994)

- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

For the pNTP303:(dsRNA-APP) chimeric gene

- a NTP303 promoter region (Wetering, 1994)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen et al., 1990)

3.5 Construction of the chimeric marker genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6:

For the *gat* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a aminoglycoside 6'-acetyltransferase encoding DNA (WO 94/26913)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

For the *bar* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a phosphinotricin acetyltransferase encoding DNA (US 5,646,024)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

3.6. Construction of the T-DNA vectors comprising the PCD modulating chimeric genes

Using appropriate restriction enzymes, the chimeric PCD modulating genes described under 3.1 to 3.5 are excised and introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (WO 97/13865) together

with either the *gat* marker gene or the *bar* marker gene. The resulting T-DNA vectors are schematically represented in Figure 6.

3.7. Introduction of the T-DNA vectors in *Agrobacterium*

The T-DNA vectors are introduced in *Agrobacterium tumefaciens* C58C1Rif(pGV4000) by electroporation as described by Walkerpeach and Velten (1995) and transformants are selected using spectinomycin and streptomycin.

Example 4. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with the T-DNA vectors of Example 3.

The *Agrobacterium* strains are used to transform *Arabidopsis thaliana* var. C24 applying the root transformation method as described by Valvekens et al. (1992). The explants are coinfected with the *Agrobacteria* strains containing the dsRNA-APP respectively the dsRNA-ZAP constructs. The dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transformants are selected for phosphinothricin resistance. The regenerated rooted transgenic lines are tested for the presence of the other T-DNA by screening for kanamycin resistance. Transgenic lines containing both T-DNA's are transfered to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

Example 5. *Agrobacterium*-mediated transformation of *Brassica napus* with the T-DNA vectors of Example 3.

The *Agrobacterium* strains are used to transform the *Brassica napus* var. N90-740 applying the hypocotyl transformation method essentially as described by De Block et *al.* (1989), except for the following modifications:

- hypocotyl explants are precultured for 1 day on A2 medium [MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.5% agarose, 1 mg/l 2,4-D, 0.25 mg/l naphthalene acetic acid (NAA)and 1 mg/l 6-benzylaminopurine (BAP)].

- infection medium A3 is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.1 mg/l NAA, 0.75 mg/l BAP and 0.01 mg/l gibberellinic acid (GA3).
- selection medium A5G is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 40 mg/l adenine.SO_4, 0.5 g/l polyvinylpyrrolidone (PVP), 0.5% agarose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l GA3, 250 mg/l carbenicillin, 250 mg/l triacillin, 5 mg/l $AgNO_3$ for three weeks. After this period selection is continued on A5J medium (similar a A5G but with 3% sucrose)
- regeneration medium A6 is MS, 0.5 g/l Mes (pH5.7), 2% sucrose, 40 mg/l adenine.SO_4, 0.5 g/l PVP, 0.5% agarose, 0.0025mg/l BAP and 250 mg/l triacillin.
- healthy shoots are transferred to rooting medium which was A9: half concentrated MS, 1,5% sucrose (pH5.8), 100 mg/l triacillin, 0.6 % agar in 1 liter vessels.

MS stands for Murashige and Skoog medium (Murashige and Skoog, 1962)

For introducing both the dsRNA-APP and the dsRNA-ZAP T-DNA constructs into a same plant cell the co-transformation method is applied, essentially as described by De Block and Debrouwer (1991). Transformed plant lines are selected on phosphinothricin containing medium after which the presence of the second T-DNA is screened by testing the regenerated rooted shoots for kanamycin resistance. In the co-transformation experiments, the dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transgenic lines containing both T-DNA's are transfered to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

Example 6. In vitro assay to test vigor of plant lines

6.1. Fitness assay for *Brassica napus*

Media and reaction buffers

Sowing medium:

Half concentrated Murashige and Skoog salts

2% sucrose

pH 5.8

0.6% agar

Callus inducing medium: A2S

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO_4, 0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP

Incubation medium:

25mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)

1 drop Tween20 for 25ml buffer

Sterilization of seeds and growing of the seedlings

Seeds are soaked in 70% ethanol for 2 min, then surface-sterilized for 15 min in a sodium hypochlorite solution (with about 6% active chlorine) containing 0.1% Tween20. Finally, the seeds are rinsed with 1l of sterile destilled water. Put 7 seeds/1l vessel (Weck) containing about 75ml of sowing medium. The seeds are germinated at 23°C and 30 µEinstein/$s^{-1}m^{-2}$ with a daylength of 16h.

The line N90-740 is always included for standardization between experiments.

Preculture of the hypocotyl explants

- 12-14 days after sowing, the hypocotyls are cut in about 7mm segments. 25 hypocotyls/Optilux Petridisch (Falcon S1005)
- The hypocotyl explants are cultured for 4 days on medium A2S at 23-25°C (at 30µEinstein/$s^{-1}m^{-2}$).
  - P.S.: about 150-300 hypocotyl explants/line are needed to cary out the asssay
- Transfer the hypocotyl explants to Optilux Petridishes (Falcon S1005) containing 30ml of incubation medium.
- Incubate for about 20hours at 24°C in the dark.

TTC-assay

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without TTC).

- Add 25ml-30ml of reaction buffer/tube.

  tube 1 ⊐ no TTC added

  * for measuring background absorption

  * one line/experiment is sufficient

  tube 2 □ +10mM TTC

  (explants have to be submerged, but do not vacuum infiltrate!)

- turn tubes upside down
- Incubate for about 1hour in the dark at 26°C (**no** end reaction!)
- Wash hypocotyls with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room°t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1hour
- Measure absorptions of extracts at 485nm

  P.S.: reduced TTC-H is not stable □ keep in the dark and measure $O.D._{485}$ as soon as possible

  $O.D._{485\ (TTC\text{-}H)} = (O.D._{485} + TTC) - (O.D._{485} - TTC)$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of N90-740 in the different experiment at 100%.
- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.

6.2. Fitness assay *Arabidopsis*

Media and reaction buffers

Plant medium: Half concentrated Murashige and Skoog salts

1.5% sucrose

pH 5.8

0.6% agar

→ autoclave 15min.

add filter sterilized -100mg/l myo-inositol

- 0.5mg/l pyridoxine
- 0.5mg/l nicotinic acid
- 1mg/l thiamine

Incubation medium: 10mM K-phosphate buffer pH5.8
2% sucrose
1 drop Tween20 for 25ml medium

Reaction buffer: 50mM K-phosphate buffer pH7.4
10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)
1 drop Tween20 for 25ml buffer

Arabidopsis plants

- Sterilization of *Arabidopsis* seeds

2min. 70% ethanol
10 min. bleach (6% active chlorine) + 1drop Tween 20 for 20ml solution
wash 5 times with sterile water

P.S.: sterilization is done in 2ml eppendorf tubes
Arabidopsis seeds sink to the bottom of the tube, allowing removal of the liquids by means of a 1ml pipetman

- Growing of *Arabidopsis* plants

Seeds are sown in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025) containing ±100ml of plant medium: 1 seed/grid.
Plants are grown at 23°C
40µEinstein $s^{-1}m^{-2}$
16hours light - 8hours dark
for about 3 weeks (plants start to form flower buds)

→ P.S.: *about 90-110 plants/line are needed to cary out the asssay
* include control line (C24; Columbia; ...) for calibration

Pre-incubation

- Harvest *Arabidopsis* shoots by cutting of roots (by means of scissors)
Put each shoot immediatly **in** incubation medium (shoots have to be submerged, but do not vacuum infiltrate)

Incubation medium: ±150ml in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025)

a) incubation medium: for quantification of background absorption (see TTC-asssay)

b) incubation medium

c) incubation medium + 2mM niacinamide

30 - 35 shoots/petridish (but same amount of shoots for all lines and for each condition)

- Incubate at 24°C in the dark for ±20hours

TTC-assay

- Transfer shoots to 50ml Falcon tubes
- Wash with reaction buffer (without TTC)
- Add 30-35ml of reaction buffer/tube

a) no TTC added (for measuring background absorption)

b and c) +10mM TTC

(Shoots have to be submerged, but do not vacuum infiltrate!)

- Incubate for about 2hours in the dark at 26°C (**no** end reaction!)
- Wash shoots with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room°t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1hour
- Measure absorptions of extracts at 485nm

P.S.: reduced TTC-H is not stable → keep in the dark and measure $O.D._{485}$ as soon as possible

- Compare reducing profiles of tested lines *versus* control line (for population of 30 to 35 plants)

$$O.D._{485\ (TTC\text{-}H)} = (O.D._{485} + TTC) - (O.D._{485} - TTC)$$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of control line (C24; Columbia; ...) in the different experiments at 100%.

- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.
- If the addition of niacinamide to the incubation medium results in a higher TTC-reducing capacity indicates to a lower fitness (as shown for C24 and Columbia).

Example 7. Phenotypic analyses of the transgenic lines containing both dsRNA-APP and dsRNA-ZAP constructs.

The flower phenotype and pollen viability (Alexander staining (Alexander, 1969) and germination asssay) of the T0-lines containing dsRNA-APP and dsRNA-ZAP under the control of tapetum or pollen specific promoters were scored. For *Arabidopsis*, the T1-generation is obtained by selving or if the plants are male sterile by backcrossing using pollen of non-transformed wild type plants. For *Brassica napus*, the T1-generation is always obtained by backcrossing using pollen of non-transformed plants.
T1-seed is germinated on kanamycin containing medium after which the resistant plants are scored by means of the ammonium-multiwell assay for phosphinothricine resistance (De Block et al., 1995). One half of the plants that contains both T-DNA's is transfered to the greenhouse to score the male fertility of the plants, while the other half is used to quantify the vigor of the plants by means of the fitness assay.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of 35S or NOS promoter, a high vigor is observed in a number of the transgenic lines.
For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of TA29 male sterility is observed in a number of the transgenic lines.
For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of NTP303 sterile pollen is observed in a number the transgenic lines.

Example 8. Phenotypic analysis of plants comprising a PCD modulating chimeric gene.

Another example of a p35S::(dsRNA-ZAP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a CaMV35S2 promoter region (Odell et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a ZAP2 encoding DNA region of Zea Mays from the HincII site to the SnaBI site having the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 1728
- the 5' end of the ZAP2 encoding region from the HincII site to the EcoRV site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 792
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG33, which was introduced in Agrobacterium C58C1Rif(pGV4000) by electroporation as described.

Another example of a pNos::(dsRNA-ZAP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a nopaline synthase promoter region (Herrera-Estrella et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a ZAP2 encoding DNA region of Zea Mays from the HincII site to the SnaBI site having the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 1728
- the 5' end of the ZAP2 encoding region from the HincII site to the EcoRV site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 792
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG34, which was introduced in Agrobacterium C58C1Rif(pGV4000) by electroporation as described.
Another example of a p35S::(dsRNA-APP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a CaMV35S2 promoter region (Odell et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a APP encoding DNA region of Arabidopsis thaliana from the ScaI site to the SmaI site having the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 1349
- the 5' end of the ZAP2 encoding region from the ScaI site to the HaeIII site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 784)
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG29, which was introduced in Agrobacterium C58C1Rif(pGV4000) by electroporation as described.

Another example of a pNos::(dsRNA-APP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a nopaline synthase promoter region (Herrera-Estrella et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a APP encoding DNA region of Arabidopsis thaliana from the ScaI site to the SmaI site having the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 1349
- the 5' end of the ZAP2 encoding region from the ScaI site to the HaeIII site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 784)
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG30, which was introduced in Agrobacterium C58C1Rif(pGV4000) by electroporation as described.

The resulting Agrobacterium strains were used to introduce the different PCD modulating genes separately into Brassica napus and Arabidopsis thaliana (Columbia and C24) plants as described in Examples 4 and 5.

Transgenic Arabidopsis thaliana plants obtained by selfing of the T0 generation (T1 generation) were germinated on medium containing phosphinotricin. The resistant transgenic plants were further cultivated.

Growth of transgenic T1 plants (both derived from Columbia or C24) comprising a pNOS::(dsRNA-ZAP) construct as in pTYG33 or a p35S::(dsRNA-ZAP) construct as in pTYG34, was significantly faster than control transgenic plants transformed by the T-DNA of the T-DNA vector without PCD modulating chimeric gene (see Table 1).

Stress tolerance of the Arabidopsis T1 transgenic plants (derived from Columbia) was evaluated by floating small plants on a salicylic acid solution of either 10 or 50 mg/L or for control just on H_2O. Stress sensitive plants developed bleached and curled leaves after 1to 2 days incubation, while stress tolerant plants remained intact for at least five days. Again transgenic plants comprising a pNOS::(dsRNA-ZAP) construct as in pTYG33 or a p35S::(dsRNA-ZAP) construct as in pTYG34, were significantly more stress-tolerant than control transgenic plants (see Table 1).

PPT-resistant transgenic callus obtained from Brassica napus transformed by the dsRNA-ZAP or dsRNA-APP constructs of pTYG29, pTYG30, pTYG33 or pTYG34, was incubated on a medium containing 50 mg/L aspirine for 2 days. After 2 days, the weight of the calli was determined and the calli were transferred on a medium without aspirine and further incubated for 5 days. At the end of the 5 days period, the weight of the calli was determined, and the increase in weight was expressed as a percentage of the weight after the two days period incubation. As a control, transgenic callus transformed by a T-DNA without a PCD modulating chimeric gene

was taken through the same procedure with the exception that no aspirine was added during the 2 day incubation. The results are summarized in Table II and indicate that transgenic Brassica napus cells comprising a PCD modulating chimeric gene are more stress resistant than the control cells.

Table 1. Evaluation of transgenic Arabidopsis plants (T1 generation)

Chimeric PCD modulating gene	Growth (Columbia and C24)	Stress tolerance (Columbia)
pNOS::(dsRNA-ZAP)	+++	++
p35S::(dsRNA-ZAP)	++	+
pNOS::(dsRNA-APP)	+	+/-
p35S::(dsRNA-APP)	+	-
Control	+	+/- (**)

** A. thalina Columbia has a certain degree of natural tolerance to aspirin.

Table 2. Regrowth of the transgenic Brassica calli after incubation on aspirine.

Chimeric PCD modulating gene	Increase in weight (%)
pNOS::(dsRNA-ZAP)	80
p35S::(dsRNA-ZAP)	90
pNOS::(dsRNA-APP)	75
p35S::(dsRNA-APP)	85
Control	70

Standard error of the mean is < 5%.

REFERENCES

Alexander (1969) Stain Technology **44**, 117

Alvarez-Gonzalez and Althaus (1989) *Mut. Res.* **218**, 67-74

An *et al.* (1996) *The Plant Cell* **8**, 15-30

An *et al.* (1996b) *Plant Journal* **10**, 107-121

Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA.

Ausubel *et al.* (1987) *Current Protocols in Molecular Biology 1987-1988.* New York: Greene Publishing Associates & Wiley-Interscience

Babiychuk *et al* (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 12722-12727

Beeckman and Engler (1994) *Plant Mol. Biol. Rep.* **12**, 37-42.

Chen *et al.* (1994) *Eur. J. Biochem* **224**, 135-142

Citovsky *et al.* (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 3210-3214.

Cohen (1993) *Immunol. Today* **14**, 126-130

Collinge and Althaus (1994) *Mol. Gen. Genet.* **245**, 686-693

Croy (1993) *Plant Molecular Biology Labfax* jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Cullin and Pompon (1988) *Gene*, **65**, 203-217

De Block *et al.* (1987) *EMBO J.* **6**, 2513-2518

De Block et al. (1989) *Plant Physiol.* **91**: 694

De Block and Debrouwer (1991) *Theor. Appl. Genet* **82**, 257-263

De Block *et al.* (1995) *Planta* **197**, 619-626

de Murcia and Ménissier de Murcia (1994) *Trends Biochem. Sci.* **19**, 172-176.

Deblaere *et al.* (1985) *Nucleic Acids Res.* **13**, 4777-4788

Depicker *et al.* (1982) *J. Mol. App. Gen.* **1**, 561-573

Ding *et al.* (1992) *J. Biol. Chem.* **267**, 12804-12812

Dohmen *et al.* (1991) *Yeast*, **7**, 691-692

Doonan *et al.* (1998) in "*Plant Cell Division*" (Francis, Duditz and Inzé, Eds.)Portland Press, London

Ellis *et al.* (1991) *Annu. Rev. Cell. Biol.* **7**, 663-698

Gallie and Walbot (1992) *Nucleic Acids Res.* **20**, 4631-4638

Gatz *et al.* (1988) *Proc. Natl. Acad. Sc. USA* **85**, 1394-1397

Gavrieli *et al.* (1992) *J. Cell. Biol.* **119**, 493-501

Harlow and Lane (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press

Harpster *et al.* (1988) *Mol. Gen. Genet.* **212**, 182-190.

Hawkins and Phillips (1983) *Plant Sci. Lett.* **32**, 221-224

Heller *et al.* (1995) *J. Biol. Chem.* **270**, 11176-11180

Herrera-Estrella et al. (1983) *EMBO J.* **2**, 987

Hudspeth *et al.* (1989) *Plant Mol Biol* **12**, 579-589

Hurkman and Tanaka (1986) *Plant Physiol.* **81**, 802-806

Ikajima *et al.* (1990) *J. Biol. Chem.* **265**, 21907-21913

Jefferson *et al.* (1987) *EMBO J.* **6**, 3901-3907

Kameshita *et al.* (1984) *J. Biol. Chem.* **259**, 4770-4776

Kawamitsu *et al.* (1984) *Biochemistry*, **23**, 3771-3777

Keil *et al.* (1989) *EMBO J.* **8**, 1323-1330

Keller *et al.* (1988) *EMBO J.* **7**, 3625-3633

Keller *et al.* (1989) *Genes Devel.* **3**, 1639-1646

Kuepper *et al.* (1990) *J. Biol. Chem.* **265**, 18721-18724

Kuge and Jones (1994) *EMBO J.* **13**, 655-664

Lazebnik *et al.* (1994) *Nature* **371**, 346-347

Lee *et al.* (1995) *Plant J.* **8**, 603-612

Lepiniec *et al.* (1995) *FEBS Letters* **364**,103-108

Lindahl *et al.* (1995) *Trends Biochem. Sci.* **20**, 405-411

Long *et al.* (1996) *Nature*, **379**, 66-69

Maliga *et al.* (1975) *Nature*, **255**, 401-402

McBride and Summerfelt (1990) *Plant Mol. Biol.* **14**, 269-276

Ménissier de Murcia *et al.* (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 7303-7307

Mogen *et al.* (1990) *The Plant Cell* **2**, 1261

Molinette *et al.* (1993) *EMBO J.* **12**, 2109-2117

Odell *et al.* (1985) *Nature* **313**, 810

O' Farrel (1995) *Biochimie* **77**, 486-491

Payne *et al.* (1976) *Exp. Cell Res.* **99**, 428-432

Peleman *et al.* (1989) *Gene* **84**, 359-369

Pennell and Lamb (1997) *The Plant Cell* **9**, 1157-1168

Phillips and Hawkins (1985) *J. Exp. Bot.* **36**, 119-128

Puchta *et al.* (1995) *Plant J.* **7**, 203-210

Sakamoto and Nagatani (1996) *Plant J.* **10**, 859-868

Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY

Schreiber *et al.* (1992) *EMBO J.* **11**, 3263-3269

Sheen et al. (1995) *The Plant Journal* **8**, 777-784

Shoji *et al.* (1997) *Plant Cell Physiol.* **38**, 36-43

Smulson *et al.* (1995) *J. Biol. Chem.* **270**, 119-127

Studier and Moffat (1986) *J. Mol. Biol.* **189**, 113-130

Sugiyama *et al.* (1995) *J. Plant Res.* **108**, 351-361

Terzaghi *et al.* (1997) *Plant J.* **11**, 967-982

Valvekens *et al.* (1988) PNAS **85**, 5536

von Arnim and Deng (1994) *Cell*, **79**, 1035-1045

Wang *et al.* (1995) *Genes Dev.* **9**, 509-520

Wang *et al.* (1996) *Plant Cell* **8**, 375-391

Wang *et al.* (1997) *Genes Dev.* **11**, 2347-2358

Weimann *et al.* (1994) *Plant J.* **5**, 559-569

Wetering (1994) PhD thesis, Katholieke Universiteis Nijmegen

Walkerpeach and Velten (1995) In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant Molecular Biology Manual pp B1/1-B1/19. Kluwer Academic Publishers, Dordrecht

Wilbur and Lipmann (1983) *Proc. Natl. Acad. Sci. USA* **80**, 706

Willmitzer and Wagner (1982) In *ADP-Ribosylation Reactions* (Hayashi, O. and Ueda, K., eds). New York: Academic Press, pp. 241-252

Zhang *et al.* (1994) *Science*, **263**, 687-689

We claim:

1. A method for modulating programmed cell death in a eukaryotic cell, said method comprising using (I) a nucleotide sequence of a poly(ADP-ribose) polymerase (PARP) gene of the ZAP class, and (II) a nucleotide sequence of a PARP gene of the NAP class to reduce the functional level of the total PARP activity in said eukaryotic cell.

2. The method of claim 1, further comprising reducing expression of PARP genes endogenous to said eukaryotic cell by using said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.

3. The method of claim 1, further comprising reducing the apparent activity of the proteins encoded by the endogenous PARP genes by using said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.

4. The method of claim 1, further comprising altering the nucleotide sequence of the endogenous PARP genes with said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.

5. A method for modulating programmed cell death (PCD) in a eukaryotic cell, comprising introducing a first and a second PCD modulating chimeric gene in said eukaryotic cell, wherein said first PCD modulating chimeric gene comprises the following operably linked DNA regions:

a) a first promoter, operative in said eukaryotic cell;
b) a first DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either
    i) capable of reducing the functional level of a Zn-finger containing PARP protein of the ZAP class; or

ii) capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of ZAP class.

c) a DNA region involved in transcription termination and polyadenylation

and wherein said second PCD modulating chimeric gene comprises the following operably linked DNA regions:

a) a second promoter, operative in said eukaryotic cell;

b) a second DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either

i) capable of reducing the functional level of a PARP protein of the NAP class; or

ii) capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of the NAP class

c) a DNA region involved in transcription termination and polyadenylation;

wherein the total apparent PARP activity in said eukaryotic cell is reduced significantly or almost completely.

6. The method of claim 5, wherein said first transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

7. The method of claim 5, wherein said second transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the NAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

8. The method of claim 7, wherein said first transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about

100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

9. The method of claim 5, wherein said first transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the DNA strand of an endogenous PARP gene of the ZAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

10. The method of claim 5, wherein said second transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the NAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

11. The method of claim 10, wherein said first transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

12. The method of claim 5, wherein said first transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the ZAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule

is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

13. The method of claim 5, wherein said second transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the NAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the NAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

14. The method of claim 10, wherein said first transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the ZAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

15. The method of claim 5, wherein said first transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP class.

16. The method of claim 5, wherein said second transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the NAP class.

17. The method of claim 16, wherein said first transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP class.

18. The method of claim 16, wherein said dominant negative PARP mutant comprises an amino acid sequence selected from the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138.

19. The method of claim 17, wherein said dominant negative PARP mutant comprises an amino acid sequence selected from the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370, the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11

20. The method of claim 5, wherein said first or said second promoter is a tissue specific or inducible promoter.

21. The method of claim 20, wherein said first or said second promoter is selected from a fungus-responsive promoter, a nematode-responsive promoter, an anther-selective promoter, a stigma-selective promoter, a dehiscence-zone selective promoter.

22. The method of any one of claim 5 to claim 21, wherein said total apparent PARP activity is reduced from about 75% to about 90% of the normal apparent PARP activity in said eukaryotic cell, and wherein said eukaryotic cell is protected against programmed cell death.

23. The method of any one of claim 5 to claim 21, wherein said total apparent PARP activity is reduced from about 90% to about 100% of the normal apparent PARP activity in said eukaryotic cell, and wherein said eukaryotic cell is killed by programmed cell death.

24. The method claim 22, wherein said eukaryotic cell is a plant cell.

25. The method of claim 23, wherein said eukaryotic cell is a plant cell.

26. A method for modulating programmed cell death in a plant cell, comprising introducing a PCD modulating chimeric gene in said plant cell, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

a) a plant-expressible promoter;
b) DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either
   i) capable of reducing the expression of endogenous PARP genes; or
   ii) capable of being translated into a peptide or protein which when expressed reduces the apparent PARP activity in said plant cell; and
c) a DNA region involved in transcription termination and polyadenylation;

wherein the total apparent PARP activity in said plant cell is reduced from about 75% to about 100% of the normal apparent PARP activity in said plant cell.

27. A first and second chimeric PCD modulating gene as claimed in any one of claims 5 to 21.

28. A eukaryotic cell comprising a first and second chimeric PCD modulating gene of claim 27.

29. The eukaryotic cell of claim 28, which is a plant cell

30. An non-human eukaryotic organism which comprises the eucaryotic cell of claim 28.

31. A plant comprising the plant cell of claim 29.

32. A seed of the plant of claim 31, comprising the first and second chimeric PCD modulating gene of claim 27.

33. A method for modulating programmed cell death in cells of a plant, said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

(a) a plant-expressible promoter;

(b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and

(c) a DNA region involved in transcription termination and polyadenylation

34. A method for increasing the growth rate of a plant, said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

(a) a plant-expressible promoter;

(b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and

(c) a DNA region involved in transcription termination and polyadenylation

35. A method for producing stress tolerant cells of a plant said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

(a) a plant-expressible promoter;

(b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and

(c) a DNA region involved in transcription termination and polyadenylation

36. Use of a nucleotide sequence encoding a protein with PARP activity to modulate programmed cell death in a plant cell or plant.

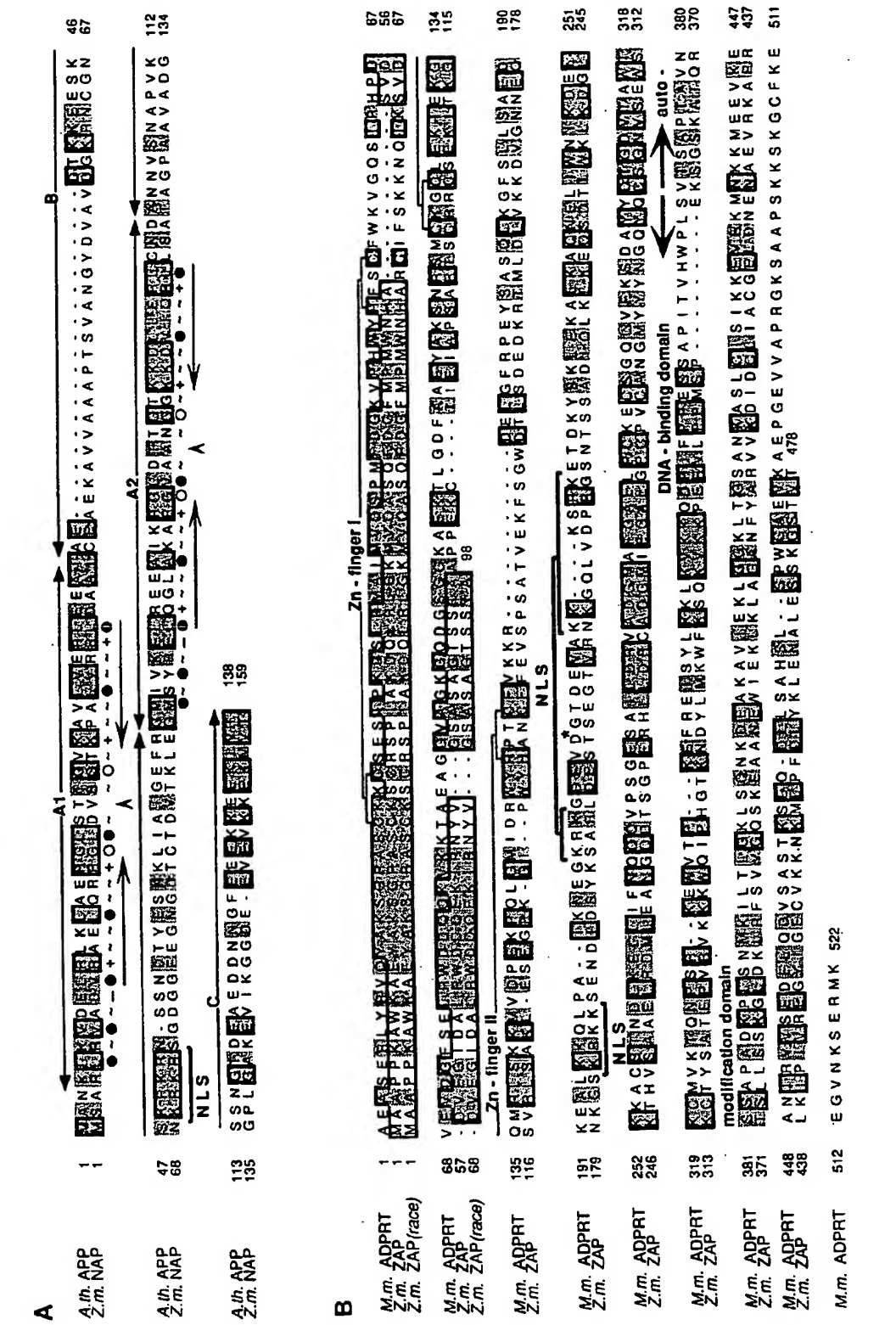
37. The use according to claim 36, wherein said protein with PARP activity is a PARP protein of the ZAP class.

38. Use of a nucleotide sequence encoding a protein with PARP activity to produce a stress tolerant plant cell or plant.

39. The use according to claim 38, wherein said protein with PARP activity is a PARP protein of the ZAP class.

40. Use of a nucleotide sequence encoding a protein with PARP activity to increase the growth rate of a plant cell or plant.

41. The use according to claim 40, wherein said protein with PARP activity is a PARP protein of the ZAP class.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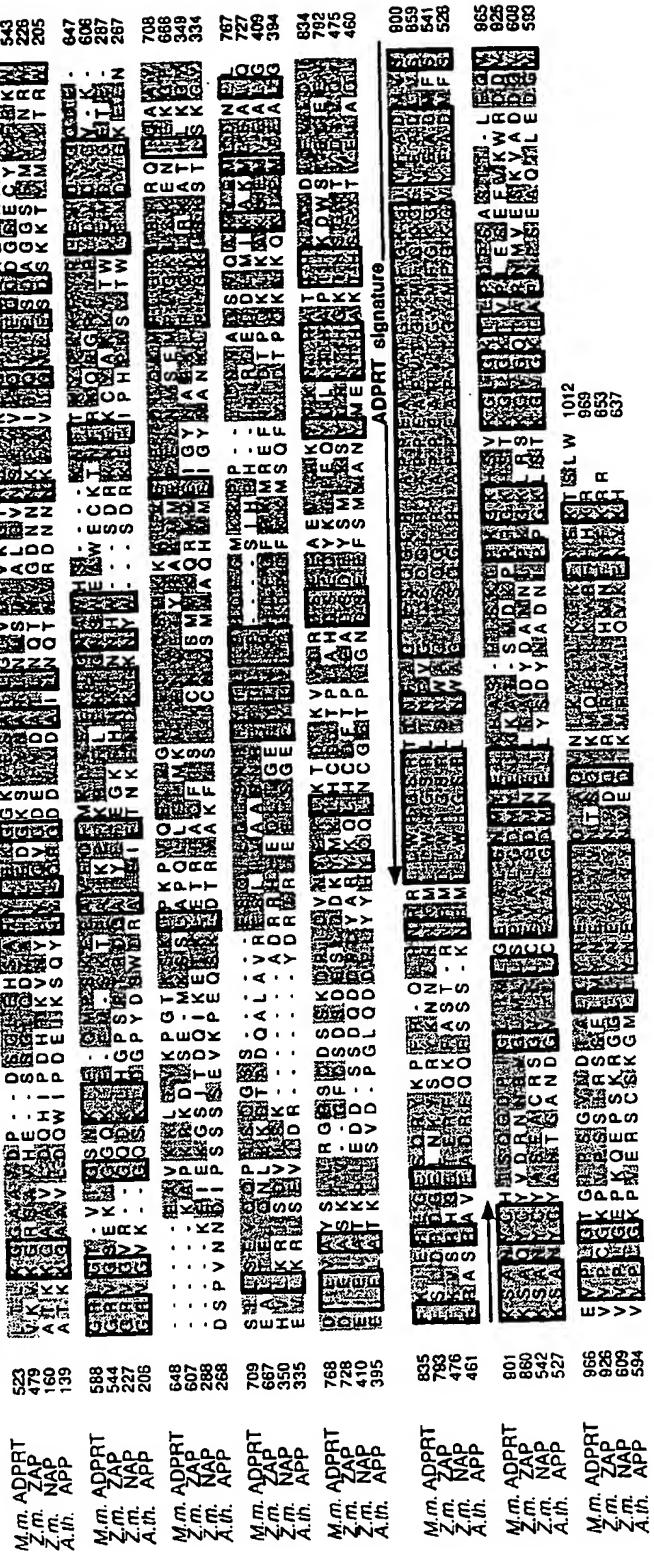

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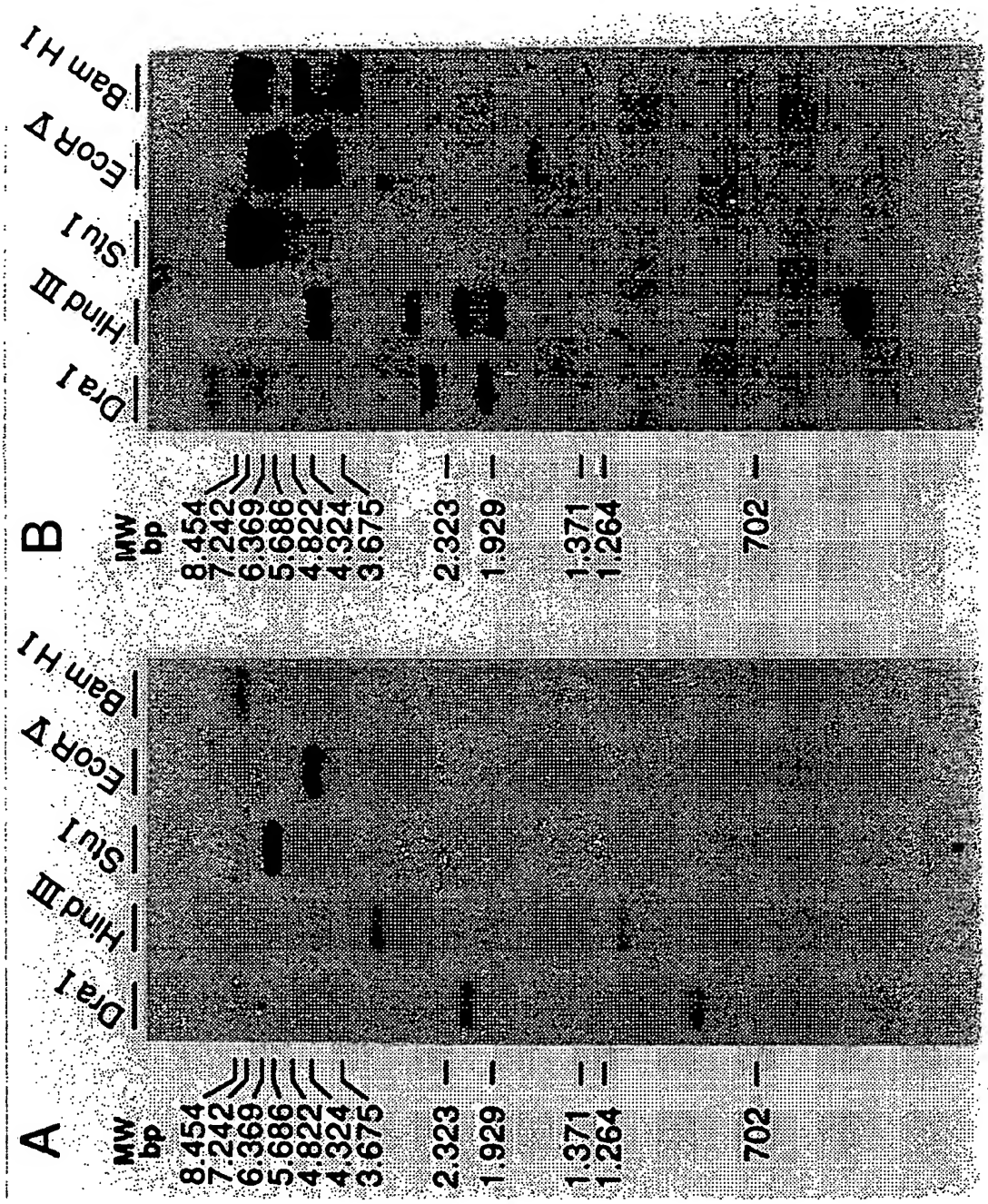
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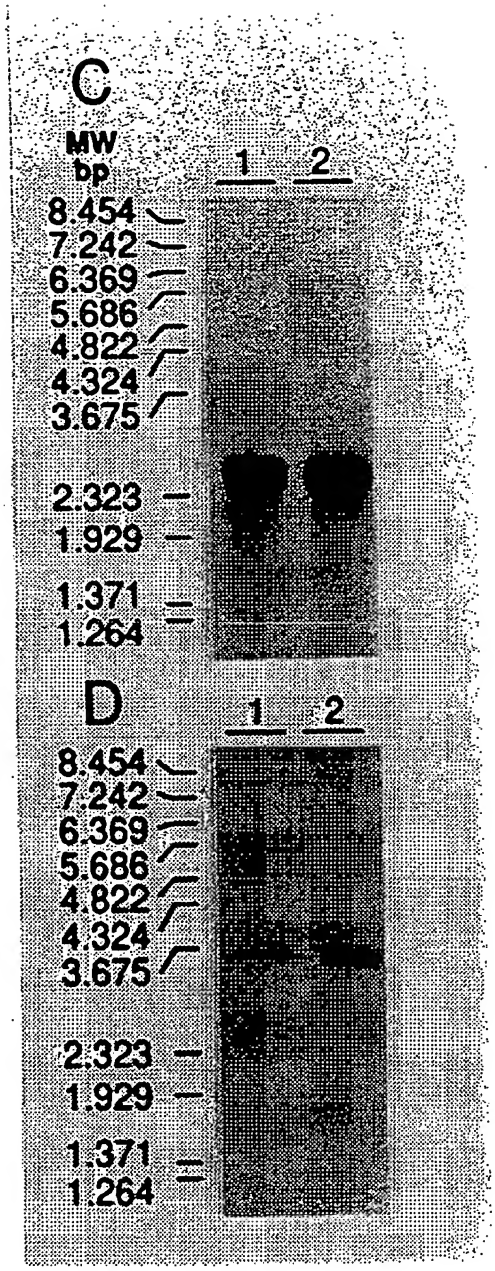

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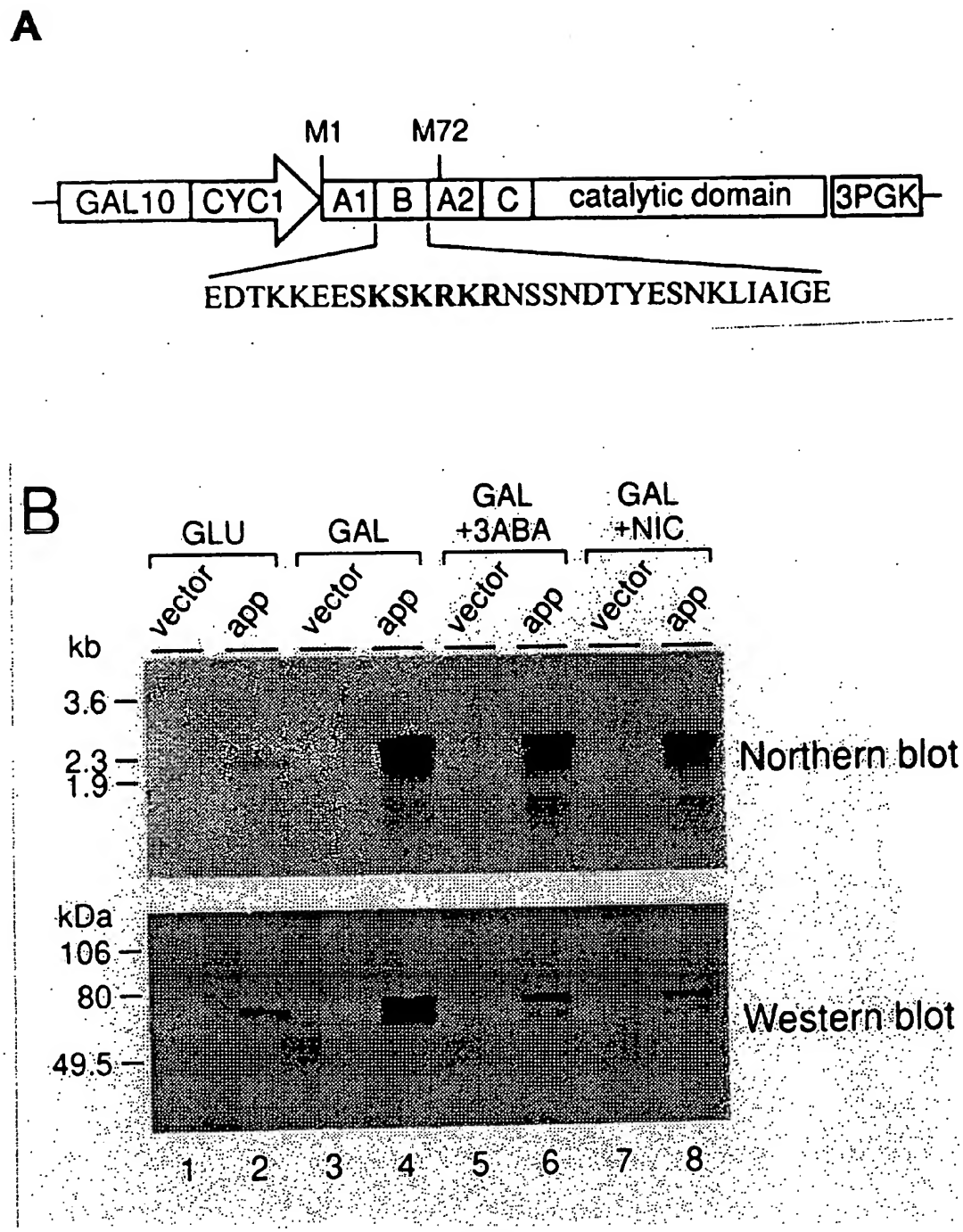
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Figure 4


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Figure 5

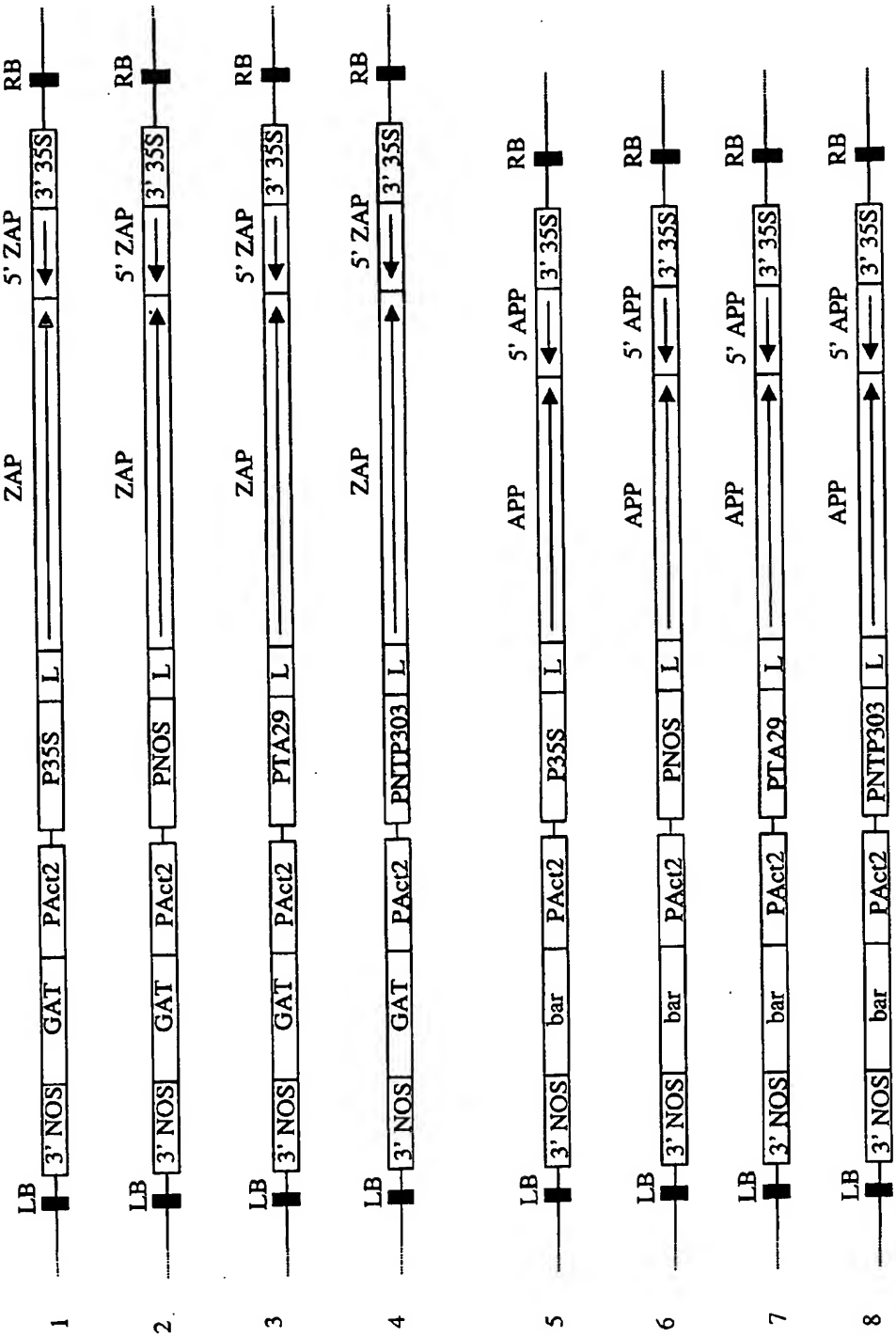
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Figure 6

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35 40 45 50

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355                 360                 365                 370

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675                 680                 685                 690

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Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser Ile His
                695                 700                 705

cct cat att ata cgg gat gag gat gat ttg atg atc aaa gcg aaa atg   2278
Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala Lys Met
            710                 715                 720

ctt gaa gct ctg cag gat att gaa att gct tca aag ata gtt ggc ttc   2326
Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val Gly Phe
        725                 730                 735

gat agc gac agt gat gaa tct ctt gat gat aaa tat atg aaa ctt cac   2374
Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys Leu His
    740                 745                 750

tgt gac atc acc ccg ctg gct cac gat agt gaa gat tac aag tta att   2422
Cys Asp Ile Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys Leu Ile
755                 760                 765                 770

gag cag tat ctc ctc aac aca cat gct cct act cac aag gac tgg tcg   2470
Glu Gln Tyr Leu Leu Asn Thr His Ala Pro Thr His Lys Asp Trp Ser
                775                 780                 785

ctg gaa ctg gag gaa gtt ttt tca ctt gat cga gat gga gaa ctt aat   2518
Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu Leu Asn
            790                 795                 800

aag tac tca aga tat aaa aat aat ctg cat aac aag atg cta tta tgg   2566
Lys Tyr Ser Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu Leu Trp
        805                 810                 815

cac ggt tca agg ttg acg aat ttt gtg gga att ctt agt caa ggg cta   2614
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His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln Gly Leu
    820                 825                 830

aga att gca cct cct gag gca cct gtt act ggc tat atg ttc ggc aaa   2662
Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly Lys
835                 840                 845                 850

ggc ctc tac ttt gca gat cta gta agc aag agc gca caa tac tgt tat   2710
Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr Cys Tyr
                855                 860                 865

gtg gat agg aat aat cct gta ggt ttg atg ctt ctt tct gag gtt gct   2758
Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu Val Ala
            870                 875                 880

tta gga gac atg tat gaa cta aag aaa gcc acg tcc atg gac aaa cct   2806
Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp Lys Pro
        885                 890                 895

cca aga ggg aag cat tcg acc aag gga tta ggc aaa acc gtg cca ctg   2854
Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val Pro Leu
    900                 905                 910

gag tca gag ttt gtg aag tgg agg gat gat gtc gta gtt ccc tgc ggc   2902
Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro Cys Gly
915                 920                 925                 930

aag ccg gtg cca tca tca att agg agc tct gaa ctc atg tac aat gag   2950
Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr Asn Glu
                935                 940                 945

tac atc gtc tac aac aca tcc cag gtg aag atg cag ttc ttg ctg aag   2998
Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu Leu Lys
            950                 955                 960

gtg cgt ttc cat cac aag agg tag ctgggagact aggcaagtag agttggaagg  3052
Val Arg Phe His His Lys Arg
        965                 970

tagagaagca gagttaggcg atgcctcttt tggtattatt agtaagcctg gcatgtattt 3112

atgggtgctc gcgcttgatc cattttggta agtgttgctt gggcatcagc gcgaatagca 3172

ccaatcacac actttacct aatgacgttt tactgtata                          3211
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Arg Ala Ser Cys Lys Ser Cys Arg Ser Pro Ile Ala Lys Asp Gln Leu
            20                  25                  30

Arg Leu Gly Lys Met Val Gln Ala Ser Gln Phe Asp Gly Phe Met Pro
        35                  40                  45

Met Trp Asn His Ala Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu
    50                  55                  60

Arg Trp Asp Asp Gln Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser
65                  70                  75                  80

Ala Gly Thr Ser Ser Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu
                85                  90                  95

Ile Ala Pro Ser Ala Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile
            100                 105                 110

Thr Lys Gly Ser Val Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro
        115                 120                 125

Lys Gly Ile Pro Trp Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro
    130                 135                 140

Ser Ala Thr Val Glu Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu
145                 150                 155                 160

Asp Lys Arg Thr Met Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn
                165                 170                 175

Glu Gln Asn Lys Gly Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp
            180                 185                 190

Ser Tyr Lys Ser Ala Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val
        195                 200                 205

Arg Asn Lys Gly Gln Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser
    210                 215                 220

Ala Asp Ile Gln Leu Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys
225                 230                 235                 240
```

```
Leu Lys Asp Gly Leu Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp
               245                 250                 255

Met Leu Glu Ala Asn Gly Gln Asp Thr Ser Gly Pro Glu Arg His Leu
           260                 265                 270

Leu Asp Arg Cys Ala Asp Gly Met Ile Phe Gly Ala Leu Gly Pro Cys
       275                 280                 285

Pro Val Cys Ala Asn Gly Met Tyr Tyr Tyr Asn Gly Gln Tyr Gln Cys
   290                 295                 300

Ser Gly Asn Val Ser Glu Trp Ser Lys Cys Thr Tyr Ser Ala Thr Glu
305                 310                 315                 320

Pro Val Arg Val Lys Lys Lys Trp Gln Ile Pro His Gly Thr Lys Asn
               325                 330                 335

Asp Tyr Leu Met Lys Trp Phe Lys Ser Gln Lys Val Lys Lys Pro Glu
           340                 345                 350

Arg Val Leu Pro Pro Met Ser Pro Glu Lys Ser Gly Ser Lys Ala Thr
       355                 360                 365

Gln Arg Thr Ser Leu Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe
   370                 375                 380

Ser Val Val Gly Gln Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys
385                 390                 395                 400

Leu Lys Leu Ala Gly Ala Asn Phe Tyr Ala Arg Val Val Lys Asp Ile
               405                 410                 415

Asp Cys Leu Ile Ala Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val
           420                 425                 430

Arg Lys Ala Arg Arg Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile
       435                 440                 445

Gly Glu Cys Val Lys Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys
   450                 455                 460

Leu Glu Asn Ala Leu Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys
465                 470                 475                 480

Val Lys Gly Arg Ser Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr
               485                 490                 495
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Ala His Ile Leu Glu Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn
500 505 510

Met Ser Asp Leu Ala Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile
515 520 525

Ile Glu Gln Asp Asp Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly
530 535 540

Arg Val Gly Ser Glu Lys Ile Gly Gly Gln Lys Leu Glu Glu Met Ser
545 550 555 560

Lys Thr Glu Ala Ile Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr
565 570 575

Gly Asn Ser Trp Glu Ala Trp Glu Cys Lys Thr Asn Phe Arg Lys Gln
580 585 590

Pro Gly Arg Phe Tyr Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala
595 600 605

Pro Lys Arg Lys Asp Ile Ser Glu Met Lys Ser Ser Leu Ala Pro Gln
610 615 620

Leu Leu Glu Leu Met Lys Met Leu Phe Asn Val Glu Thr Tyr Arg Ala
625 630 635 640

Ala Met Met Glu Phe Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys
645 650 655

Leu Ser Lys Glu Asn Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile
660 665 670

Gln Asn Leu Leu Lys Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu
675 680 685

Ser Leu Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser
690 695 700

Ile His Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala
705 710 715 720

Lys Met Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val
725 730 735

Gly Phe Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys
740 745 750

Leu His Cys Asp Ile Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys
755 760 765

Leu Ile Glu Gln Tyr Leu Leu Asn Thr His Ala Pro Thr His Lys Asp
770 775 780

Trp Ser Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu
785 790 795 800

Leu Asn Lys Tyr Ser Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu
805 810 815

Leu Trp His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln
820 825 830

Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe
835 840 845

Gly Lys Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr
850 855 860

Cys Tyr Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu
865 870 875 880

Val Ala Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp
885 890 895

Lys Pro Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val
900 905 910

Pro Leu Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro
915 920 925

Cys Gly Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr
930 935 940

Asn Glu Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu
945 950 955 960

Leu Lys Val Arg Phe His His Lys Arg
965

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<213> Zea mays

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Met Ser Ala
1

agg cta cgg gtg gcg gac gtc cgc gcg gag ctt cag cgc cgc ggc ctc 163
Arg Leu Arg Val Ala Asp Val Arg Ala Glu Leu Gln Arg Arg Gly Leu
5 10 15

gat gta tcc ggc acc aag cct gct ctc gtg cgg agg ctg gac gcc gca 211
Asp Val Ser Gly Thr Lys Pro Ala Leu Val Arg Arg Leu Asp Ala Ala
20 25 30 35

att tgc gag gcg gag aag gcc gtg gtg gct gct gcg cca acc agt gtg 259
Ile Cys Glu Ala Glu Lys Ala Val Val Ala Ala Ala Pro Thr Ser Val
40 45 50

gca aat ggg tat gac gta gcc gta gat ggc aaa agg aac tgc ggg aat 307
Ala Asn Gly Tyr Asp Val Ala Val Asp Gly Lys Arg Asn Cys Gly Asn
55 60 65

aat aag agg aaa agg tcc ggg gat ggg ggt gaa gag gga aac ggc gat 355
Asn Lys Arg Lys Arg Ser Gly Asp Gly Gly Glu Glu Gly Asn Gly Asp
70 75 80

acg tgt aca gat gtg aca aaa cta gag ggc atg agc tat cgt gag ctg 403
Thr Cys Thr Asp Val Thr Lys Leu Glu Gly Met Ser Tyr Arg Glu Leu
85 90 95

cag gga ttg gcc aag gca cgt gga gtt gcg gca aat ggg ggc aag aaa 451
Gln Gly Leu Ala Lys Ala Arg Gly Val Ala Ala Asn Gly Gly Lys Lys
100 105 110 115

gat gtt atc cag agg ttg ctc tcg gcg act gct ggt cct gct gca gtt 499
Asp Val Ile Gln Arg Leu Leu Ser Ala Thr Ala Gly Pro Ala Ala Val
120 125 130

gca gat ggt ggt cct ctg ggc gcc aag gaa gtc ata aaa ggt ggt gat 547
Ala Asp Gly Gly Pro Leu Gly Ala Lys Glu Val Ile Lys Gly Gly Asp
135 140 145

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gag gag gtt gag gtg aaa aag gag aag atg gtt act gcc acg aag aag   595
Glu Glu Val Glu Val Lys Lys Glu Lys Met Val Thr Ala Thr Lys Lys
        150                 155                 160

gga gct gca gtg ctg gat cag cac att ccc gat cac ata aaa gtg aac   643
Gly Ala Ala Val Leu Asp Gln His Ile Pro Asp His Ile Lys Val Asn
    165                 170                 175

tat cat gtc ttg caa gtg ggc gat gaa atc tat gat gcc acc ttg aac   691
Tyr His Val Leu Gln Val Gly Asp Glu Ile Tyr Asp Ala Thr Leu Asn
180                 185                 190                 195

cag act aat gtt gga gac aac aac aat aag ttc tat atc att caa gtt   739
Gln Thr Asn Val Gly Asp Asn Asn Asn Lys Phe Tyr Ile Ile Gln Val
                200                 205                 210

tta gaa tct gat gct ggt gga agc ttt atg gtt tac aat aga tgg gga   787
Leu Glu Ser Asp Ala Gly Gly Ser Phe Met Val Tyr Asn Arg Trp Gly
            215                 220                 225

aga gtt ggg gta cga ggt caa gat aaa cta cat ggt ccc tcc cca aca   835
Arg Val Gly Val Arg Gly Gln Asp Lys Leu His Gly Pro Ser Pro Thr
        230                 235                 240

cga gac caa gca ata tat gaa ttt gag ggg aag ttc cac aac aaa acc   883
Arg Asp Gln Ala Ile Tyr Glu Phe Glu Gly Lys Phe His Asn Lys Thr
    245                 250                 255

aat aat cat tgg tct gat cgc aag aac ttc aaa tgt tat gca aag aaa   931
Asn Asn His Trp Ser Asp Arg Lys Asn Phe Lys Cys Tyr Ala Lys Lys
260                 265                 270                 275

tac act tgg ctt gaa atg gat tat ggt gaa act gag aaa gaa ata gag   979
Tyr Thr Trp Leu Glu Met Asp Tyr Gly Glu Thr Glu Lys Glu Ile Glu
                280                 285                 290

aaa ggt tcc att act gat cag ata aaa gag aca aaa ctt gaa act aga   1027
Lys Gly Ser Ile Thr Asp Gln Ile Lys Glu Thr Lys Leu Glu Thr Arg
            295                 300                 305

att gcg cag ttc ata tcc ctg atc tgc aat att agc atg atg aag caa   1075
Ile Ala Gln Phe Ile Ser Leu Ile Cys Asn Ile Ser Met Met Lys Gln
        310                 315                 320

aga atg gtg gaa ata ggt tat aat gct gaa aag ctt ccc ctt gga aag   1123
Arg Met Val Glu Ile Gly Tyr Asn Ala Glu Lys Leu Pro Leu Gly Lys
    325                 330                 335
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cta agg aaa gct aca ata ctt aag ggt tat cat gtt ttg aaa agg ata   1171
Leu Arg Lys Ala Thr Ile Leu Lys Gly Tyr His Val Leu Lys Arg Ile
340                 345                 350                 355

tcc gat gtt att tca aag gcg gac agg aga cat ctt gag caa ttg act   1219
Ser Asp Val Ile Ser Lys Ala Asp Arg Arg His Leu Glu Gln Leu Thr
                360                 365                 370

ggg gaa ttc tac acc gtg att cct cat gac ttt ggt ttc aga aag atg   1267
Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly Phe Arg Lys Met
            375                 380                 385

cgt gaa ttt att atc gat act cct cag aaa cta aaa gct aag ttg gag   1315
Arg Glu Phe Ile Ile Asp Thr Pro Gln Lys Leu Lys Ala Lys Leu Glu
        390                 395                 400

atg gtt gaa gcc ctt ggt gag att gaa att gca act aaa ctt ttg gag   1363
Met Val Glu Ala Leu Gly Glu Ile Glu Ile Ala Thr Lys Leu Leu Glu
    405                 410                 415

gat gat tca agt gac cag gat gat ccg ttg tat gct cga tac aag caa   1411
Asp Asp Ser Ser Asp Gln Asp Asp Pro Leu Tyr Ala Arg Tyr Lys Gln
420                 425                 430                 435

ctt cat tgt gat ttc aca cct ctt gaa gct gat tca gat gag tac tct   1459
Leu His Cys Asp Phe Thr Pro Leu Glu Ala Asp Ser Asp Glu Tyr Ser
                440                 445                 450

atg ata aaa tca tat ttg aga aat aca cat gga aaa aca cac ttt ggt   1507
Met Ile Lys Ser Tyr Leu Arg Asn Thr His Gly Lys Thr His Ser Gly
            455                 460                 465

tat acg gtg gac ata gtg caa ata ttt aag gtt tca agg cat ggt gaa   1555
Tyr Thr Val Asp Ile Val Gln Ile Phe Lys Val Ser Arg His Gly Glu
        470                 475                 480

aca gag cga ttt caa aaa ttt gct agt aca aga aat agg atg ctt ttg   1603
Thr Glu Arg Phe Gln Lys Phe Ala Ser Thr Arg Asn Arg Met Leu Leu
    485                 490                 495

tgg cat ggt tct cgg ttg agc aac tgg gct ggg atc ctt tct cag ggt   1651
Trp His Gly Ser Arg Leu Ser Asn Trp Ala Gly Ile Leu Ser Gln Gly
500                 505                 510                 515

ctg cga atc gct cct cct gaa gca cct gtt act ggt tac atg ttt ggc   1699
Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly
                520                 525                 530
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aag ggt gtt tac ttt gct gac atg ttt tca aag agt gca aac tat tgc   1747
Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser Ala Asn Tyr Cys
            535                 540                 545

tac gcc tct gaa gca tgt aga tct gga gta ctg ctt tta tgt gag gtt   1795
Tyr Ala Ser Glu Ala Cys Arg Ser Gly Val Leu Leu Leu Cys Glu Val
        550                 555                 560

gca ttg ggc gat atg aat gag cta ctg aat gca gat tac gat gct aat   1843
Ala Leu Gly Asp Met Asn Glu Leu Leu Asn Ala Asp Tyr Asp Ala Asn
    565                 570                 575

aac ctg ccc aaa gga aaa tta aga tcc aag gga gtt ggt caa aca gca   1891
Asn Leu Pro Lys Gly Lys Leu Arg Ser Lys Gly Val Gly Gln Thr Ala
580                 585                 590                 595

cct aac atg gtc gag tct aag gtc gct gac gat ggt gtt gtt gtt ccc   1939
Pro Asn Met Val Glu Ser Lys Val Ala Asp Asp Gly Val Val Val Pro
                600                 605                 610

ctt ggc gaa ccc aaa cag gaa cct tcc aaa agg ggt ggc ttg ctt tat   1987
Leu Gly Glu Pro Lys Gln Glu Pro Ser Lys Arg Gly Gly Leu Leu Tyr
            615                 620                 625

aat gag tac ata gtg tac aac gta gac cag ata aga atg cgg tat gtc   2035
Asn Glu Tyr Ile Val Tyr Asn Val Asp Gln Ile Arg Met Arg Tyr Val
        630                 635                 640

tta cat gtt aac ttc aat ttc aag aga cgg tag atgttgcaaa gagctgaaac 2088
Leu His Val Asn Phe Asn Phe Lys Arg Arg
    645                 650

tgttgctgag atcttagcag aacatatgtg gacttatagc accaggtgcc ctcagcctca 2148

ttttctgagc aaatttggta gcctttgcat ttcgattttg gtttcagctt ctagccccat 2208

tgatgattga tactgagtgt atatatgaac cattgatatc caccttccat gtacttaagt 2268

ttttttaaca tgtcccatgc ataataa                                     2295
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            20                  25                  30

Asp Ala Ala Ile Cys Glu Ala Glu Lys Ala Val Val Ala Ala Ala Pro
        35                  40                  45

Thr Ser Val Ala Asn Gly Tyr Asp Val Ala Val Asp Gly Lys Arg Asn
    50                  55                  60

Cys Gly Asn Asn Lys Arg Lys Arg Ser Gly Asp Gly Gly Glu Glu Gly
65                  70                  75                  80

Asn Gly Asp Thr Cys Thr Asp Val Thr Lys Leu Glu Gly Met Ser Tyr
                85                  90                  95

Arg Glu Leu Gln Gly Leu Ala Lys Ala Arg Gly Val Ala Ala Asn Gly
            100                 105                 110

Gly Lys Lys Asp Val Ile Gln Arg Leu Leu Ser Ala Thr Ala Gly Pro
        115                 120                 125

Ala Ala Val Ala Asp Gly Gly Pro Leu Gly Ala Lys Glu Val Ile Lys
    130                 135                 140

Gly Gly Asp Glu Glu Val Glu Val Lys Lys Glu Lys Met Val Thr Ala
145                 150                 155                 160

Thr Lys Lys Gly Ala Ala Val Leu Asp Gln His Ile Pro Asp His Ile
                165                 170                 175

Lys Val Asn Tyr His Val Leu Gln Val Gly Asp Glu Ile Tyr Asp Ala
            180                 185                 190

Thr Leu Asn Gln Thr Asn Val Gly Asp Asn Asn Asn Lys Phe Tyr Ile
        195                 200                 205

Ile Gln Val Leu Glu Ser Asp Ala Gly Gly Ser Phe Met Val Tyr Asn
    210                 215                 220

Arg Trp Gly Arg Val Gly Val Arg Gly Gln Asp Lys Leu His Gly Pro
225                 230                 235                 240

Ser Pro Thr Arg Asp Gln Ala Ile Tyr Glu Phe Glu Gly Lys Phe His
                245                 250                 255

Asn Lys Thr Asn Asn His Trp Ser Asp Arg Lys Asn Phe Lys Cys Tyr
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260 265 270

Ala Lys Lys Tyr Thr Trp Leu Glu Met Asp Tyr Gly Glu Thr Glu Lys
275 280 285

Glu Ile Glu Lys Gly Ser Ile Thr Asp Gln Ile Lys Glu Thr Lys Leu
290 295 300

Glu Thr Arg Ile Ala Gln Phe Ile Ser Leu Ile Cys Asn Ile Ser Met
305 310 315 320

Met Lys Gln Arg Met Val Glu Ile Gly Tyr Asn Ala Glu Lys Leu Pro
325 330 335

Leu Gly Lys Leu Arg Lys Ala Thr Ile Leu Lys Gly Tyr His Val Leu
340 345 350

Lys Arg Ile Ser Asp Val Ile Ser Lys Ala Asp Arg Arg His Leu Glu
355 360 365

Gln Leu Thr Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly Phe
370 375 380

Arg Lys Met Arg Glu Phe Ile Ile Asp Thr Pro Gln Lys Leu Lys Ala
385 390 395 400

Lys Leu Glu Met Val Glu Ala Leu Gly Glu Ile Glu Ile Ala Thr Lys
405 410 415

Leu Leu Glu Asp Asp Ser Ser Asp Gln Asp Asp Pro Leu Tyr Ala Arg
420 425 430

Tyr Lys Gln Leu His Cys Asp Phe Thr Pro Leu Glu Ala Asp Ser Asp
435 440 445

Glu Tyr Ser Met Ile Lys Ser Tyr Leu Arg Asn Thr His Gly Lys Thr
450 455 460

His Ser Gly Tyr Thr Val Asp Ile Val Gln Ile Phe Lys Val Ser Arg
465 470 475 480

His Gly Glu Thr Glu Arg Phe Gln Lys Phe Ala Ser Thr Arg Asn Arg
485 490 495

Met Leu Leu Trp His Gly Ser Arg Leu Ser Asn Trp Ala Gly Ile Leu
500 505 510

Ser Gln Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr

515 520 525

Met Phe Gly Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser Ala
530 535 540

Asn Tyr Cys Tyr Ala Ser Glu Ala Cys Arg Ser Gly Val Leu Leu Leu
545 550 555 560

Cys Glu Val Ala Leu Gly Asp Met Asn Glu Leu Leu Asn Ala Asp Tyr
565 570 575

Asp Ala Asn Asn Leu Pro Lys Gly Lys Leu Arg Ser Lys Gly Val Gly
580 585 590

Gln Thr Ala Pro Asn Met Val Glu Ser Lys Val Ala Asp Asp Gly Val
595 600 605

Val Val Pro Leu Gly Glu Pro Lys Gln Glu Pro Ser Lys Arg Gly Gly
610 615 620

Leu Leu Tyr Asn Glu Tyr Ile Val Tyr Asn Val Asp Gln Ile Arg Met
625 630 635 640

Arg Tyr Val Leu His Val Asn Phe Asn Phe Lys Arg Arg
645 650

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<222> (129)..(2042)

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acgaaaacca tacttcctca gtctcattcc ctttccgacg aactattctc ctgaagaaga 120

agacgaaa atg gcg aac aag ctc aaa gtc gac gaa ctc cgt tta aaa ctc 170
Met Ala Asn Lys Leu Lys Val Asp Glu Leu Arg Leu Lys Leu
1 5 10

gcc gag cgt gga ctc agt act act gga gtc aaa gcc gtt ctg gtg gag 218
Ala Glu Arg Gly Leu Ser Thr Thr Gly Val Lys Ala Val Leu Val Glu
15 20 25 30

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agg ctt gaa gag gct atc gca gaa gac act aag aag gaa gaa tca aag   266
Arg Leu Glu Glu Ala Ile Ala Glu Asp Thr Lys Lys Glu Glu Ser Lys
                35                  40                  45

agc aag agg aaa aga aat tct tct aat gat act tat gaa tcg aac aaa   314
Ser Lys Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys
            50                  55                  60

ttg att gca att ggc gaa ttt cgt ggg atg att gtg aag gaa ttg cgt   362
Leu Ile Ala Ile Gly Glu Phe Arg Gly Met Ile Val Lys Glu Leu Arg
        65                  70                  75

gag gaa gct att aag aga ggc tta gat aca aca gga acc aaa aag gat   410
Glu Glu Ala Ile Lys Arg Gly Leu Asp Thr Thr Gly Thr Lys Lys Asp
    80                  85                  90

ctt ctt gag agg ctt tgc aat gat gct aat aac gtt tcc aat gca cca   458
Leu Leu Glu Arg Leu Cys Asn Asp Ala Asn Asn Val Ser Asn Ala Pro
95                  100                 105                 110

gtc aaa tcc agt aat ggg aca gat gaa gct gaa gat gac aac aat ggc   506
Val Lys Ser Ser Asn Gly Thr Asp Glu Ala Glu Asp Asp Asn Asn Gly
                115                 120                 125

ttt gaa gaa gaa aag aaa gaa gag aaa atc gta acc gcg aca aag aag   554
Phe Glu Glu Glu Lys Lys Glu Glu Lys Ile Val Thr Ala Thr Lys Lys
            130                 135                 140

ggt gca gcg gtg tta gat cag tgg att cct gat gag ata aag agt cag   602
Gly Ala Ala Val Leu Asp Gln Trp Ile Pro Asp Glu Ile Lys Ser Gln
        145                 150                 155

tac cat gtt cta caa agg ggt gat gat gtt tat gat gct atc tta aat   650
Tyr His Val Leu Gln Arg Gly Asp Asp Val Tyr Asp Ala Ile Leu Asn
    160                 165                 170

cag aca aat gtc agg gat aat aat aac aag ttc ttt gtc cta caa gtc   698
Gln Thr Asn Val Arg Asp Asn Asn Asn Lys Phe Phe Val Leu Gln Val
175                 180                 185                 190

cta gag tcg gat agt aaa aag aca tac atg gtt tac act aga tgg gga   746
Leu Glu Ser Asp Ser Lys Lys Thr Tyr Met Val Tyr Thr Arg Trp Gly
                195                 200                 205

aga gtt ggt gtg aaa gga caa agt aag cta gat ggg cct tat gac tca   794
Arg Val Gly Val Lys Gly Gln Ser Lys Leu Asp Gly Pro Tyr Asp Ser
            210                 215                 220
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tgg gat cgt gcg ata gag ata ttt acc aat aag ttc aat gac aag aca   842
Trp Asp Arg Ala Ile Glu Ile Phe Thr Asn Lys Phe Asn Asp Lys Thr
        225                 230                 235

aag aat tat tgg tct gac aga aag gag ttt atc cca cat ccc aag tcc   890
Lys Asn Tyr Trp Ser Asp Arg Lys Glu Phe Ile Pro His Pro Lys Ser
    240                 245                 250

tat aca tgg ctc gaa atg gat tac gga aaa gag gaa aat gat tca ccg   938
Tyr Thr Trp Leu Glu Met Asp Tyr Gly Lys Glu Glu Asn Asp Ser Pro
255                 260                 265                 270

gtc aat aat gat att ccg agt tca tct tcc gaa gtt aaa cct gaa caa   986
Val Asn Asn Asp Ile Pro Ser Ser Ser Ser Glu Val Lys Pro Glu Gln
                275                 280                 285

tca aaa cta gat act cgg gtt gcc aag ttc atc tct ctt ata tgt aat   1034
Ser Lys Leu Asp Thr Arg Val Ala Lys Phe Ile Ser Leu Ile Cys Asn
            290                 295                 300

gtc agc atg atg gca cag cat atg atg gaa ata gga tat aac gct aac   1082
Val Ser Met Met Ala Gln His Met Met Glu Ile Gly Tyr Asn Ala Asn
        305                 310                 315

aaa ttg cca ctc ggc aag ata agc aag tcc aca att tca aag ggt tat   1130
Lys Leu Pro Leu Gly Lys Ile Ser Lys Ser Thr Ile Ser Lys Gly Tyr
    320                 325                 330

gaa gtg ctg aag aga ata tcg gag gtg att gac cgg tat gat aga acg   1178
Glu Val Leu Lys Arg Ile Ser Glu Val Ile Asp Arg Tyr Asp Arg Thr
335                 340                 345                 350

agg ctt gag gaa ctg agt gga gag ttc tac aca gtg ata cct cat gat   1226
Arg Leu Glu Glu Leu Ser Gly Glu Phe Tyr Thr Val Ile Pro His Asp
                355                 360                 365

ttt ggt ttt aag aaa atg agt cag ttt gtt ata gac act cct caa aag   1274
Phe Gly Phe Lys Lys Met Ser Gln Phe Val Ile Asp Thr Pro Gln Lys
            370                 375                 380

ttg aaa cag aaa att gaa atg gtt gaa gca tta ggt gaa att gaa ctc   1322
Leu Lys Gln Lys Ile Glu Met Val Glu Ala Leu Gly Glu Ile Glu Leu
        385                 390                 395

gca aca aag ttg ttg tcc gtc gac ccg gga ttg cag gat gat cct tta   1370
Ala Thr Lys Leu Leu Ser Val Asp Pro Gly Leu Gln Asp Asp Pro Leu
    400                 405                 410
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tat tat cac tac cag caa ctt aat tgt ggt ttg acg cca gta gga aat   1418
Tyr Tyr His Tyr Gln Gln Leu Asn Cys Gly Leu Thr Pro Val Gly Asn
415                 420                 425                 430

gat tca gag gag ttc tct atg gtt gct aat tac atg gag aac act cat   1466
Asp Ser Glu Glu Phe Ser Met Val Ala Asn Tyr Met Glu Asn Thr His
                435                 440                 445

gca aag acg cat tcg gga tat acg gtt gag att gcc caa cta ttt aga   1514
Ala Lys Thr His Ser Gly Tyr Thr Val Glu Ile Ala Gln Leu Phe Arg
        450                 455                 460

gct tcg aga gct gtt gaa gct gat cga ttc caa cag ttt tca agt tcg   1562
Ala Ser Arg Ala Val Glu Ala Asp Arg Phe Gln Gln Phe Ser Ser Ser
    465                 470                 475

aag aac agg atg tta ctc tgg cac ggt tca cgt ctc act aac tgg gct   1610
Lys Asn Arg Met Leu Leu Trp His Gly Ser Arg Leu Thr Asn Trp Ala
480                 485                 490

ggt att tta tct caa ggt ctg cga ata gct cct cct gaa gcg cct gta   1658
Gly Ile Leu Ser Gln Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val
495                 500                 505                 510

act ggt tac atg ttt gga aaa ggg gtt tac ttt gcg gat atg ttc tcc   1706
Thr Gly Tyr Met Phe Gly Lys Gly Val Tyr Phe Ala Asp Met Phe Ser
                515                 520                 525

aag agt gcg aac tat tgc tat gcc aac act ggc gct aat gat ggc gtt   1754
Lys Ser Ala Asn Tyr Cys Tyr Ala Asn Thr Gly Ala Asn Asp Gly Val
            530                 535                 540

ctg ctc ctc tgc gag gtt gct ttg gga gac atg aat gaa ctt ctg tat   1802
Leu Leu Leu Cys Glu Val Ala Leu Gly Asp Met Asn Glu Leu Leu Tyr
        545                 550                 555

tca gat tat aac gcg gat aat cta ccc ccg gga aag cta agc aca aaa   1850
Ser Asp Tyr Asn Ala Asp Asn Leu Pro Pro Gly Lys Leu Ser Thr Lys
    560                 565                 570

ggt gtg ggg aaa aca gca cca aac cca tca gag gct caa aca cta gaa   1898
Gly Val Gly Lys Thr Ala Pro Asn Pro Ser Glu Ala Gln Thr Leu Glu
575                 580                 585                 590

gac ggt gtt gtt gtt cca ctt ggc aaa cca gtg gaa cgt tca tgc tcc   1946
Asp Gly Val Val Val Pro Leu Gly Lys Pro Val Glu Arg Ser Cys Ser
                595                 600                 605
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aag ggg atg ttg ttg tac aac gaa tat ata gtc tac aat gtg gaa caa 1994
Lys Gly Met Leu Leu Tyr Asn Glu Tyr Ile Val Tyr Asn Val Glu Gln
610 615 620

atc aag atg cgt tat gtg atc caa gtc aaa ttc aac tac aag cac taa 2042
Ile Lys Met Arg Tyr Val Ile Gln Val Lys Phe Asn Tyr Lys His
625 630 635

aacttatgta tattagcttt tgaacatcaa ctaattatcc aaaaatcagc gttttattgt 2102

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35 40 45

Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys Leu Ile
50 55 60

Ala Ile Gly Glu Phe Arg Gly Met Ile Val Lys Glu Leu Arg Glu Glu
65 70 75 80

Ala Ile Lys Arg Gly Leu Asp Thr Thr Gly Thr Lys Lys Asp Leu Leu
85 90 95

Glu Arg Leu Cys Asn Asp Ala Asn Asn Val Ser Asn Ala Pro Val Lys
100 105 110

Ser Ser Asn Gly Thr Asp Glu Ala Glu Asp Asp Asn Asn Gly Phe Glu
115 120 125

Glu Glu Lys Lys Glu Glu Lys Ile Val Thr Ala Thr Lys Lys Gly Ala
130 135 140

Ala Val Leu Asp Gln Trp Ile Pro Asp Glu Ile Lys Ser Gln Tyr His

145 150 155 160

Val Leu Gln Arg Gly Asp Asp Val Tyr Asp Ala Ile Leu Asn Gln Thr
165 170 175

Asn Val Arg Asp Asn Asn Asn Lys Phe Phe Val Leu Gln Val Leu Glu
180 185 190

Ser Asp Ser Lys Lys Thr Tyr Met Val Tyr Thr Arg Trp Gly Arg Val
195 200 205

Gly Val Lys Gly Gln Ser Lys Leu Asp Gly Pro Tyr Asp Ser Trp Asp
210 215 220

Arg Ala Ile Glu Ile Phe Thr Asn Lys Phe Asn Asp Lys Thr Lys Asn
225 230 235 240

Tyr Trp Ser Asp Arg Lys Glu Phe Ile Pro His Pro Lys Ser Tyr Thr
245 250 255

Trp Leu Glu Met Asp Tyr Gly Lys Glu Glu Asn Asp Ser Pro Val Asn
260 265 270

Asn Asp Ile Pro Ser Ser Ser Ser Glu Val Lys Pro Glu Gln Ser Lys
275 280 285

Leu Asp Thr Arg Val Ala Lys Phe Ile Ser Leu Ile Cys Asn Val Ser
290 295 300

Met Met Ala Gln His Met Met Glu Ile Gly Tyr Asn Ala Asn Lys Leu
305 310 315 320

Pro Leu Gly Lys Ile Ser Lys Ser Thr Ile Ser Lys Gly Tyr Glu Val
325 330 335

Leu Lys Arg Ile Ser Glu Val Ile Asp Arg Tyr Asp Arg Thr Arg Leu
340 345 350

Glu Glu Leu Ser Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly
355 360 365

Phe Lys Lys Met Ser Gln Phe Val Ile Asp Thr Pro Gln Lys Leu Lys
370 375 380

Gln Lys Ile Glu Met Val Glu Ala Leu Gly Glu Ile Glu Leu Ala Thr
385 390 395 400

Lys Leu Leu Ser Val Asp Pro Gly Leu Gln Asp Asp Pro Leu Tyr Tyr

405 410 415

His Tyr Gln Gln Leu Asn Cys Gly Leu Thr Pro Val Gly Asn Asp Ser
420 425 430

Glu Glu Phe Ser Met Val Ala Asn Tyr Met Glu Asn Thr His Ala Lys
435 440 445

Thr His Ser Gly Tyr Thr Val Glu Ile Ala Gln Leu Phe Arg Ala Ser
450 455 460

Arg Ala Val Glu Ala Asp Arg Phe Gln Gln Phe Ser Ser Ser Lys Asn
465 470 475 480

Arg Met Leu Leu Trp His Gly Ser Arg Leu Thr Asn Trp Ala Gly Ile
485 490 495

Leu Ser Gln Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly
500 505 510

Tyr Met Phe Gly Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser
515 520 525

Ala Asn Tyr Cys Tyr Ala Asn Thr Gly Ala Asn Asp Gly Val Leu Leu
530 535 540

Leu Cys Glu Val Ala Leu Gly Asp Met Asn Glu Leu Leu Tyr Ser Asp
545 550 555 560

Tyr Asn Ala Asp Asn Leu Pro Pro Gly Lys Leu Ser Thr Lys Gly Val
565 570 575

Gly Lys Thr Ala Pro Asn Pro Ser Glu Ala Gln Thr Leu Glu Asp Gly
580 585 590

Val Val Val Pro Leu Gly Lys Pro Val Glu Arg Ser Cys Ser Lys Gly
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610 615 620

Met Arg Tyr Val Ile Gln Val Lys Phe Asn Tyr Lys His
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Xaa Xaa Xaa Gly Val Lys Xaa Xaa Leu Val Xaa Arg Leu Xaa Xaa Ala
20 25 30

Ile

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Met Ala Ala Pro Pro Lys Ala Trp Lys Ala Glu
1 5 10

tat gcc aag tct ggg cgg gcc tcg tgc aag tca tgc cgg tcc cct atc 161
Tyr Ala Lys Ser Gly Arg Ala Ser Cys Lys Ser Cys Arg Ser Pro Ile
15 20 25

gcc aag gac cag ctc cgt ctt ggc aag atg gtt cag gcg tca cag ttc 209
Ala Lys Asp Gln Leu Arg Leu Gly Lys Met Val Gln Ala Ser Gln Phe
30 35 40

gac ggc ttc atg ccg atg tgg aac cat gcc agg tgc atc ttc agc aag 257
Asp Gly Phe Met Pro Met Trp Asn His Ala Arg Cys Ile Phe Ser Lys
45 50 55

aag aac cag ata aaa tcc gtt gac gat gtt gaa ggg ata gat gca ctt 305
Lys Asn Gln Ile Lys Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu
60 65 70 75

aga tgg gat gat caa gag aag ata cga aac tac gtt ggg agt gcc tca 353
Arg Trp Asp Asp Gln Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser
80 85 90

gct ggt aca agt tct aca gct gct cct cct gag aaa tgt aca att gag 401
Ala Gly Thr Ser Ser Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu
95 100 105

att gct cca tct gcc cgt act tca tgt aga cga tgc agt gaa aag att 449
Ile Ala Pro Ser Ala Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile
110 115 120

aca aaa gga tcg gtc cgt ctt tca gct aag ctt gag agt gaa ggt ccc 497
Thr Lys Gly Ser Val Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro
125 130 135

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aag ggt ata cca tgg tat cat gcc aac tgt ttc ttt gag gta tcc ccg   545
Lys Gly Ile Pro Trp Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro
140                 145                 150                 155

tct gca act gtt gag aag ttc tca ggc tgg gat act ttg tcc gat gag   593
Ser Ala Thr Val Glu Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu
                160                 165                 170

gat aag aga acc atg ctc gat ctt gtt aaa aaa gat gtt ggc aac aat   641
Asp Lys Arg Thr Met Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn
            175                 180                 185

gaa caa aat aag ggt tcc aag cgc aag aaa agt gaa aat gat att gat   689
Glu Gln Asn Lys Gly Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp
        190                 195                 200

agc tac aaa tcc gcc agg tta gat gaa agt aca tct gaa ggt aca gtg   737
Ser Tyr Lys Ser Ala Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val
    205                 210                 215

cga aac aaa ggg caa ctt gta gac cca cgt ggt tcc aat act agt tca   785
Arg Asn Lys Gly Gln Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser
220                 225                 230                 235

gct gat atc caa cta aag ctt aag gag caa agt gac aca ctt tgg aag   833
Ala Asp Ile Gln Leu Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys
                240                 245                 250

tta aag gat gga ctt aag act cat gta tcg gct gct gaa tta agg gat   881
Leu Lys Asp Gly Leu Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp
            255                 260                 265

atg ctt gag gct aat ggg cag gat aca tca gga cca gaa agg cac cta   929
Met Leu Glu Ala Asn Gly Gln Asp Thr Ser Gly Pro Glu Arg His Leu
        270                 275                 280

ttg gat cgc tgt gcg gat gga atg ata ttt gga gcg ctg ggt cct tgc   977
Leu Asp Arg Cys Ala Asp Gly Met Ile Phe Gly Ala Leu Gly Pro Cys
    285                 290                 295

cca gtc tgt gct aat ggc atg tac tat tat aat ggt cag tac caa tgc   1025
Pro Val Cys Ala Asn Gly Met Tyr Tyr Tyr Asn Gly Gln Tyr Gln Cys
300                 305                 310                 315

agt ggt aat gtg tca gag tgg tcc aag tgt aca tac tct gcc aca gaa   1073
Ser Gly Asn Val Ser Glu Trp Ser Lys Cys Thr Tyr Ser Ala Thr Glu
                320                 325                 330
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cct gtc cgc gtt aag aag aag tgg caa att cca cat gga aca aag aat   1121
Pro Val Arg Val Lys Lys Lys Trp Gln Ile Pro His Gly Thr Lys Asn
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gat tac ctt atg aag tgg ttc aaa tct caa aag gtt aag aaa cca gag   1169
Asp Tyr Leu Met Lys Trp Phe Lys Ser Gln Lys Val Lys Lys Pro Glu
        350                 355                 360

agg gtt ctt cca cca atg tca cct gag aaa tct gga agt aaa gca act   1217
Arg Val Leu Pro Pro Met Ser Pro Glu Lys Ser Gly Ser Lys Ala Thr
    365                 370                 375

cag aga aca tca ttg ctg tct tct aaa ggg ttg gat aaa tta agg ttt   1265
Gln Arg Thr Ser Leu Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe
380                 385                 390                 395

tct gtt gta gga caa tca aaa gaa gca gca aat gag tgg att gag aag   1313
Ser Val Val Gly Gln Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys
                400                 405                 410

ctc aaa ctt gct ggt gcc aac ttc tat gcc agg gtt gtc aaa gat att   1361
Leu Lys Leu Ala Gly Ala Asn Phe Tyr Ala Arg Val Val Lys Asp Ile
            415                 420                 425

gat tgt tta att gca tgt ggt gag ctc gac aat gaa aat gct gaa gtc   1409
Asp Cys Leu Ile Ala Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val
        430                 435                 440

agg aaa gca agg agg ctg aag ata cca att gta agg gag ggt tac att   1457
Arg Lys Ala Arg Arg Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile
    445                 450                 455

gga gaa tgt gtt aaa aag aac aaa atg ctg cca ttt gat ttg tat aaa   1505
Gly Glu Cys Val Lys Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys
460                 465                 470                 475

cta gag aat gcc tta gag tcc tca aaa ggc agt act gtc act gtt aaa   1553
Leu Glu Asn Ala Leu Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys
                480                 485                 490

gtt aag ggc cga agt gct gtt cat gag tcc tct ggt ttg caa gat act   1601
Val Lys Gly Arg Ser Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr
            495                 500                 505

gct cac att ctt gaa gat ggg aaa agc ata tac aat gca acc tta aac   1649
Ala His Ile Leu Glu Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn
        510                 515                 520
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atg tct gac ctg gca cta ggt gtg aac agc tac tat gta ctc cag atc   1697
Met Ser Asp Leu Ala Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile
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att gaa cag gat gat ggg tct gag tgc tac gta ttt cgt aag tgg gga   1745
Ile Glu Gln Asp Asp Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly
540                 545                 550                 555

cgg gtt ggg agt gag aaa att gga ggg caa aaa ctg gag gag atg tca   1793
Arg Val Gly Ser Glu Lys Ile Gly Gly Gln Lys Leu Glu Glu Met Ser
                560                 565                 570

aaa act gag gca atc aag gaa ttc aaa aga tta ttt ctt gag aag act   1841
Lys Thr Glu Ala Ile Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr
            575                 580                 585

gga aac tca tgg gaa gct tgg gaa tgt aaa acc aat ttt cgg aag cag   1889
Gly Asn Ser Trp Glu Ala Trp Glu Cys Lys Thr Asn Phe Arg Lys Gln
        590                 595                 600

cct ggg aga ttt tac cca ctt gat gtt gat tat ggt gtt aag aaa gca   1937
Pro Gly Arg Phe Tyr Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala
    605                 610                 615

cca aaa cgg aaa gat atc agt gaa atg aaa agt tct ctt gct cct caa   1985
Pro Lys Arg Lys Asp Ile Ser Glu Met Lys Ser Ser Leu Ala Pro Gln
620                 625                 630                 635

ttg cta gaa ctc atg aag atg ctt ttc aat gtg gag aca tat aga gct   2033
Leu Leu Glu Leu Met Lys Met Leu Phe Asn Val Glu Thr Tyr Arg Ala
                640                 645                 650

gct atg atg gaa ttt gaa att aat atg tca gaa atg cct ctt ggg aag   2081
Ala Met Met Glu Phe Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys
            655                 660                 665

cta agc aag gaa aat att gag aaa gga ttt gaa gca tta act gag ata   2129
Leu Ser Lys Glu Asn Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile
        670                 675                 680

cag aat tta ttg aag gac acc gct gat caa gca ctg gct gtt aga gaa   2177
Gln Asn Leu Leu Lys Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu
    685                 690                 695

agc tta att gtt gct gcg agc aat cgc ttt ttc act ctt atc cct tct   2225
Ser Leu Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser
700                 705                 710                 715
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att cat cct cat att ata cgg gat gag gat gat ttg atg atc aaa gcg   2273
Ile His Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala
                720                 725                 730

aaa atg ctt gaa gct ctg cag gat att gaa att gct tca aag ata gtt   2321
Lys Met Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val
            735                 740                 745

ggc ttc gat agc gac agt gat gaa tct ctt gat gat aaa tat atg aaa   2369
Gly Phe Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys
        750                 755                 760

ctt cac tgt gac atc acc ccg ctg gct cac gat agt gaa gat tac aag   2417
Leu His Cys Asp Ile Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys
    765                 770                 775

tta att gag cag tat ctc ctc aac aca cat gct cct act cac aag gac   2465
Leu Ile Glu Gln Tyr Leu Leu Asn Thr His Ala Pro Thr His Lys Asp
780                 785                 790                 795

tgg tcg ctg gaa ctg gag gaa gtt ttt tca ctt gat cga gat gga gaa   2513
Trp Ser Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu
                800                 805                 810

ctt aat aag tac tca aga tat aaa aat aat ctg cat aac aag atg cta   2561
Leu Asn Lys Tyr Ser Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu
            815                 820                 825

tta tgg cac ggt tca agg ttg acg aat ttt gtg gga att ctt agt caa   2609
Leu Trp His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln
        830                 835                 840

ggg cta aga att gca cct cct gag gca cct gtt act ggc tat atg ttc   2657
Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe
    845                 850                 855

ggc aaa ggc ctc tac ttt gca gat cta gta agc aag agc gca caa tac   2705
Gly Lys Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr
860                 865                 870                 875

tgt tat gtg gat agg aat aat cct gta ggt ttg atg ctt ctt tct gag   2753
Cys Tyr Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu
                880                 885                 890

gtt gct tta gga gac atg tat gaa cta aag aaa gcc acg tcc atg gac   2801
Val Ala Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp
            895                 900                 905
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aaa cct cca aga ggg aag cat tcg acc aag gga tta ggc aaa acc gtg 2849
Lys Pro Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val
910 915 920

cca ctg gag tca gag ttt gtg aag tgg agg gat gat gtc gta gtt ccc 2897
Pro Leu Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro
925 930 935

tgc ggc aag ccg gtg cca tca tca att agg agc tct gaa ctc atg tac 2945
Cys Gly Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr
940 945 950 955

aat gag tac atc gtc tac aac aca tcc cag gtg aag atg cag ttc ttg 2993
Asn Glu Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu
960 965 970

ctg aag gtg cgt ttc cat cac aag agg tagctgggag actaggcaag 3040
Leu Lys Val Arg Phe His His Lys Arg
975 980

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ctggcatgta tttatgggtg ctcgcgcttg atccattttg gtaagtgttg cttgggcatc 3160

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35 40 45

Met Trp Asn His Ala Arg Cys Ile Phe Ser Lys Lys Asn Gln Ile Lys
50 55 60

Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu Arg Trp Asp Asp Gln
65 70 75 80

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Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser Ala Gly Thr Ser Ser
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Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu Ile Ala Pro Ser Ala
            100                 105                 110

Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile Thr Lys Gly Ser Val
        115                 120                 125

Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro Lys Gly Ile Pro Trp
    130                 135                 140

Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro Ser Ala Thr Val Glu
145                 150                 155                 160

Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu Asp Lys Arg Thr Met
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Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn Glu Gln Asn Lys Gly
            180                 185                 190

Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp Ser Tyr Lys Ser Ala
        195                 200                 205

Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val Arg Asn Lys Gly Gln
    210                 215                 220

Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser Ala Asp Ile Gln Leu
225                 230                 235                 240

Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys Leu Lys Asp Gly Leu
                245                 250                 255

Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp Met Leu Glu Ala Asn
            260                 265                 270

Gly Gln Asp Thr Ser Gly Pro Glu Arg His Leu Leu Asp Arg Cys Ala
        275                 280                 285

Asp Gly Met Ile Phe Gly Ala Leu Gly Pro Cys Pro Val Cys Ala Asn
    290                 295                 300

Gly Met Tyr Tyr Tyr Asn Gly Gln Tyr Gln Cys Ser Gly Asn Val Ser
305                 310                 315                 320

Glu Trp Ser Lys Cys Thr Tyr Ser Ala Thr Glu Pro Val Arg Val Lys
                325                 330                 335
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Lys Lys Trp Gln Ile Pro His Gly Thr Lys Asn Asp Tyr Leu Met Lys
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Trp Phe Lys Ser Gln Lys Val Lys Lys Pro Glu Arg Val Leu Pro Pro
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Met Ser Pro Glu Lys Ser Gly Ser Lys Ala Thr Gln Arg Thr Ser Leu
    370                 375                 380

Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe Ser Val Val Gly Gln
385                 390                 395                 400

Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys Leu Lys Leu Ala Gly
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Ala Asn Phe Tyr Ala Arg Val Val Lys Asp Ile Asp Cys Leu Ile Ala
            420                 425                 430

Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val Arg Lys Ala Arg Arg
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Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile Gly Glu Cys Val Lys
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Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys Leu Glu Asn Ala Leu
465                 470                 475                 480

Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys Val Lys Gly Arg Ser
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Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr Ala His Ile Leu Glu
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Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn Met Ser Asp Leu Ala
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Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile Ile Glu Gln Asp Asp
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Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly Arg Val Gly Ser Glu
545                 550                 555                 560

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Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr Gly Asn Ser Trp Glu
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Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala Pro Lys Arg Lys Asp
    610                 615                 620

Ile Ser Glu Met Lys Ser Ser Leu Ala Pro Gln Leu Leu Glu Leu Met
625                 630                 635                 640

Lys Met Leu Phe Asn Val Glu Thr Tyr Arg Ala Ala Met Met Glu Phe
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Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys Leu Ser Lys Glu Asn
            660                 665                 670

Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile Gln Asn Leu Leu Lys
        675                 680                 685

Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu Ser Leu Ile Val Ala
    690                 695                 700

Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser Ile His Pro His Ile
705                 710                 715                 720

Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala Lys Met Leu Glu Ala
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Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val Gly Phe Asp Ser Asp
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Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys Leu His Cys Asp Ile
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Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys Leu Ile Glu Gln Tyr
    770                 775                 780

Leu Leu Asn Thr His Ala Pro Thr His Lys Asp Trp Ser Leu Glu Leu
785                 790                 795                 800

Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu Leu Asn Lys Tyr Ser
                805                 810                 815

Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu Leu Trp His Gly Ser
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Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln Gly Leu Arg Ile Ala
        835                 840                 845
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Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly Lys Gly Leu Tyr
    850                 855                 860

Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr Cys Tyr Val Asp Arg
865                 870                 875                 880

Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu Val Ala Leu Gly Asp
                885                 890                 895

Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp Lys Pro Pro Arg Gly
            900                 905                 910

Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val Pro Leu Glu Ser Glu
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Phe Val Lys Trp Arg Asp Asp Val Val Val Pro Cys Gly Lys Pro Val
    930                 935                 940

Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr Asn Glu Tyr Ile Val
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His His Lys Arg
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<213> Artificial Sequence

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Glu Glu Ala Ile Ala Glu Asp Thr Lys Lys Glu Glu Ser Lys Ser Lys
         35                  40                  45

Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys Leu Ile
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65 70 75 80

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85 90 95

Glu Arg Leu Cys Asn Asp Ala Asn Asn Val Ser Asn Ala Pro Val Lys
100 105 110

Ser Ser Asn Gly Thr Asp Glu Ala Glu Asp Asp Asn Asn Gly Phe Glu
115 120 125

Glu Glu Lys Lys Glu Glu Lys Ile Val Thr Ala Thr Lys Lys Gly Ala
130 135 140

Ala Val Leu Asp Gln Trp Ile Pro Asp Glu Ile Lys Ser Gln Tyr His
145 150 155 160

Val Leu Gln Arg Gly Asp Asp Val Tyr Asp Ala Ile Leu Asn Gln Thr
165 170 175

Asn Val Arg Asp Asn Asn Asn Lys Phe Phe Val Leu Gln Val Leu Glu
180 185 190

Ser Asp Ser Lys Lys Thr Tyr Met Val Tyr Thr Arg Trp Gly Arg Val
195 200 205

Gly Val Lys Gly Gln Ser Lys Leu Asp Gly Pro Tyr Asp Ser Trp Asp
210 215 220

Arg Ala Ile Glu Ile Phe Thr Asn Lys Phe Asn Asp Lys Thr Lys Asn
225 230 235 240

Tyr Trp Ser Asp Arg Lys Glu Phe Ile Pro His Pro Lys Ser Tyr Thr
245 250 255

Trp Leu Glu Met Asp Tyr Gly Lys Glu Glu Asn Asp Ser Pro Val Asn
260 265 270

Asn Asp Ile Pro Ser Ser Ser Ser Glu Val Lys Pro Glu Gln Ser Lys
275 280 285

Leu Asp Thr Arg Val Ala Lys Phe Ile Ser Leu Ile Cys Asn Val Ser
290 295 300

Met Met Ala Gln His Met Met Glu Ile Gly Tyr Asn Ala Asn Lys Leu

305 310 315 320

Pro Leu Gly Lys Ile Ser Lys Ser Thr Ile Ser Lys Gly Tyr Glu Val
325 330 335

Leu Lys Arg Ile Ser Glu Val Ile Asp Arg Tyr Asp Arg Thr Arg Leu
340 345 350

Glu Glu Leu Ser Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly
355 360 365

Phe Lys Lys Met Ser Gln Phe Val Ile Asp Thr Pro Gln Lys Leu Lys
370 375 380

Gln Lys Ile Glu Met Val Glu Ala Leu Gly Glu Ile Glu Leu Ala Thr
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Lys Leu Leu Ser Val Asp Pro Met Val Arg Pro Val Glu Thr Pro Thr
405 410 415

Arg Glu Ile Lys Lys Leu Asp Gly Leu Trp Ala Phe Ser Leu Asp Arg
420 425 430

Glu Asn Cys Gly Ile Asp Gln Arg Trp Trp Glu Ser Ala Leu Gln Glu
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Ser Arg Ala Ile Ala Val Pro Gly Ser Phe Asn Asp Gln Phe Ala Asp
450 455 460

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Phe Ile Pro Lys Gly Trp Ala Gly Gln Arg Ile Val Leu Arg Phe Asp
485 490 495

Ala Val Thr His Tyr Gly Lys Val Trp Val Asn Asn Gln Glu Val Met
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Glu His Gln Gly Gly Tyr Thr Pro Phe Glu Ala Asp Val Thr Pro Tyr
515 520 525

Val Ile Ala Gly Lys Ser Val Arg Ile Thr Val Cys Val Asn Asn Glu
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Leu Asn Trp Gln Thr Ile Pro Pro Gly Met Val Ile Thr Asp Glu Asn
545 550 555 560

Gly Lys Lys Lys Gln Ser Tyr Phe His Asp Phe Phe Asn Tyr Ala Gly

565 570 575

Ile His Arg Ser Val Met Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp
580 585 590

Asp Ile Thr Val Val Thr His Val Ala Gln Asp Cys Asn His Ala Ser
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Val Asp Trp Gln Val Val Ala Asn Gly Asp Val Ser Val Glu Leu Arg
610 615 620

Asp Ala Asp Gln Gln Val Val Ala Thr Gly Gln Gly Thr Ser Gly Thr
625 630 635 640

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645 650 655

Tyr Glu Leu Cys Val Thr Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr
660 665 670

Pro Leu Arg Val Gly Ile Arg Ser Val Ala Val Lys Gly Glu Gln Phe
675 680 685

Leu Ile Asn His Lys Pro Phe Tyr Phe Thr Gly Phe Gly Arg His Glu
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Asp His Ala Leu Met Asp Trp Ile Gly Ala Asn Ser Tyr Arg Thr Ser
725 730 735

His Tyr Pro Tyr Ala Glu Glu Met Leu Asp Trp Ala Asp Glu His Gly
740 745 750

Ile Val Val Ile Asp Glu Thr Ala Ala Val Gly Phe Asn Leu Ser Leu
755 760 765

Gly Ile Gly Phe Glu Ala Gly Asn Lys Pro Lys Glu Leu Tyr Ser Glu
770 775 780

Glu Ala Val Asn Gly Glu Thr Gln Gln Ala His Leu Gln Ala Ile Lys
785 790 795 800

Glu Leu Ile Ala Arg Asp Lys Asn His Pro Ser Val Val Met Trp Ser
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Ile Ala Asn Glu Pro Asp Thr Arg Pro Gln Gly Ala Arg Glu Tyr Phe

820 825 830

Ala Pro Leu Ala Glu Ala Thr Arg Lys Leu Asp Pro Thr Arg Pro Ile
835 840 845

Thr Cys Val Asn Val Met Phe Cys Asp Ala His Thr Asp Thr Ile Ser
850 855 860

Asp Leu Phe Asp Val Leu Cys Leu Asn Arg Tyr Tyr Gly Trp Tyr Val
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Gln Ser Gly Asp Leu Glu Thr Ala Glu Lys Val Leu Glu Lys Glu Leu
885 890 895

Leu Ala Trp Gln Glu Lys Leu His Gln Pro Ile Ile Ile Thr Glu Tyr
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Gly Val Asp Thr Leu Ala Gly Leu His Ser Met Tyr Thr Asp Met Trp
915 920 925

Ser Glu Glu Tyr Gln Cys Ala Trp Leu Asp Met Tyr His Arg Val Phe
930 935 940

Asp Arg Val Ser Ala Val Val Gly Glu Gln Val Trp Asn Phe Ala Asp
945 950 955 960

Phe Ala Thr Ser Gln Gly Ile Leu Arg Val Gly Gly Asn Lys Lys Gly
965 970 975

Ile Phe Thr Arg Asp Arg Lys Pro Lys Ser Ala Ala Phe Leu Leu Gln
980 985 990

Lys Arg Trp Thr Gly Met Asn Phe Gly Glu Lys Pro Gln Gln Gly Gly
995 1000 1005

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1010

<210> 13
<211> 25
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<223> Description of Artificial Sequence: degenerated PCR primer

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<210> 14
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<220>
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<210> 16
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<210> 17
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<400> 18
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<210> 19
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fusion with beta-glucuronidase gene

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<222> (1962)..(1964)
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agccttttctc tgtaatttgg tttaagaaaa gtttttgcat tttatgtata aacgtgtttt 240

ttttttataa tttcaaattt caacaaaaaa caatttttt taataatgat tgaccactat 300

agacaattta aatgataaaa aaaaggggga atttttcaca atgttttgga gattagtcta 360

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cttaatgatt tgtactcaca atatctttta aatttaaaat tgttaaccaa aatatcctat 480

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agttaaaaca tatgtttttt ttagtatgtt ctaatcacac ctattaaaaa aagttgaagc 600

taaatgagcc aaaaagaaaa ataaagatag gggatgggga caggctgtaa tgttaggcgg 660

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/EP 99/04940

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/54 C12N9/10 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEPINIEC, L., ET AL. : "characterization of an Arabidopsis thaliana cDNA homologue to animal poly (ADP ribose) polymerase" FEBS LETTERS, vol. 364, 1995, pages 103-108, XP002102933 the whole document ---	1-41
A	MÉNISSIER DE MURCIA, J., ET AL. : "requirement of poly (ADP ribose) polymerase in revovery from DNA damage in mice and in cells" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 94, July 1997 (1997-07), pages 7303-7307, XP002120253 page 7306-7307 abstract ---	1-41
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1999	10/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

1

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/EP 99/04940

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KUEPPER J ET AL: "MOLECULAR GENETIC SYSTEMS TO STUDY THE ROLE OF POLY(ADP-RIBOSYL)ATION IN THE CELLULAR RESPONSE TO DNA DAMAGE" BIOCHIMIE, vol. 77, no. 6, 1995, pages 450-455, XP000789938 ISSN: 0300-9084 the whole document ---	1-41
A	WO 97 06267 A (PLANT GENETIC SYSTEMS NV ;BLOCK MARC DE (BE)) 20 February 1997 (1997-02-20) abstract, page 4 + 5 ---	1-41
A	LAUTIER D ET AL: "MOLECULAR AND BIOCHEMICAL FEATURES OF POLY (ADP-RIBOSE) METALBOLISM" MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 122, no. 2, 26 May 1993 (1993-05-26), pages 171-193, XP000560778 ISSN: 0300-8177 the whole document ---	1-41
A	JEGGO, P.A. : "dna repair: PARP - another guardian angel ??" CURRENT BIOLOGY, vol. 8, no. 2, January 1998 (1998-01), pages r49-51, XP002118902 the whole document ---	1-41
P,X	AMOR,Y., ET AL. : "the involvment of poly (ADP ribose) polymerase in the oxidative stress responses in plants" FEBS LETTERS , vol. 440, November 1998 (1998-11), pages 1-7, XP002102936 the whole document ---	26,36-41
P,X	MAHAJAN, P.B. AND ZUO,Z.: "purifictaion and cDNA cloning of maize poly (ADP)-ribose polymerase" PLANT PHYSIOLOGY, vol. 118, November 1998 (1998-11), pages 895-905, XP002102934 especially page 904, left column the whole document ---	36-41
E	WO 99 37789 A (PIONEER HI BRED INT ;MAHAJAN PRAMOD (US); ZUO ZHUANG (US)) 29 July 1999 (1999-07-29) abstract; page 11,12,16,24 claims --- -/--	26,33-41

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 99/04940

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	BABIYCHUK,E., ET AL. : "higher plants possess two structurally different poly (ADP ribose) polymerases" THE PLANT JOURNAL, vol. 15, no. 5, September 1998 (1998-09), pages 635-645, XP002102932 the whole document	1-41

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Interr -nal Application No
PCT/EP 99/04940

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9706267 A	20-02-1997	EP 0757102 A	05-02-1997
		AU 6739896 A	05-03-1997
		CA 2200496 A	20-02-1997
		EP 0795019 A	17-09-1997
		JP 11500627 T	19-01-1999
WO 9937789 A	29-07-1999	NONE	